Ribitol Dehydrogenase from *Klebsiella aerogenes*

PURIFICATION AND SUBUNIT STRUCTURE

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Ribitol dehydrogenase has been purified to homogeneity from several strains of *Klebsiella aerogenes*. One strain yields 3–6 g of pure enzyme from 1 kg of cells. The enzyme is a tetramer of four subunits, mol.wt. 27000. Preliminary studies of the activity of the enzyme are reported. Peptide ‘maps’ together with the amino acid composition indicate that the subunits are identical.

Klebsiella aerogenes grows on ribitol or D-arabitol as sole carbon and energy source, but not on xylitol or L-arabitol. Specific inducible dehydrogenases exist for ribitol and D-arabitol but not for the other pentitols (Mortlock *et al.*, 1965). However, mutants that are constitutive for ribitol dehydrogenase (ribitol–NAD⁺ 2-oxidoreductase, EC 1.1.1.56) grow weakly on xylitol by using a side-specificity of this enzyme (Lerner *et al.*, 1964). The K_{app} for xylitol is around 1 M compared with 1 mM for ribitol. Hence this enzyme can be considered a bad ‘xylitol dehydrogenase’ which limits the growth of the organism on this substrate (Wu *et al.*, 1968).

If the organism is grown on xylitol in continuous culture, the steady-state biomass within the chemostat vessel is controlled by the intracellular activity of this enzyme. Mutational improvements in the activity of the enzyme towards xylitol allow takeover by the ‘evolvant’ from the ancestral strain and are signalled by increases in the steady-state biomass within the vessel. We have described elsewhere a study of the evolution of the organism under this selective pressure (Hartley *et al.*, 1972; Hartley, 1974).

Previous purifications of ribitol dehydrogenase from *Kl. aerogenes* strains have yielded highly purified products, but there was some doubt about their homogeneity (Fromm, 1958; Nordlie & Fromm, 1959; Fromm & Bietz, 1966; Wu *et al.*, 1968). Large quantities of pure enzyme were needed for subsequent amino acid sequence studies. We report here proce-

dures that yield approximately 500 mg of pure enzyme from 1 kg of wet cells of the wild-type or from 100 g of cells of the ‘evolvant’ strain. Determination of the molecular weight, subunit structure and amino acid composition of this enzyme are described, together with preliminary studies of its properties. Details of the amino acid sequence and kinetic studies of its catalytic activity compared with that of a mutant with increased activity towards xylitol have been described briefly elsewhere (Hartley *et al.*, 1972; Hartley, 1974).

Materials

Bacterial strains

Strains derived from *Kl. aerogenes* 1033 (Neidhart & Magasanik, 1956) were obtained from Professor E. C. C. Lin, Department of Microbiology, Harvard Medical School, Boston, Mass. 02115, U.S.A. Strain 5P14 (*arg*[−], *gua*[−]) required arginine and guanine for growth and strain X1 (*arg*[−], *gua*[−], *R*^c) was a mutant derived from this which was constitutive (*R*^c) for the synthesis of ribitol dehydrogenase. Strains 1A (*gua*[−], *R*^c), 2A (*arg*[−], *R*^c) and A (*R*^c) were selected as spontaneous revertants of strain X1, and strains 2A1 and A11 were ‘evolvants’ of strain 2A or A which make large quantities of ribitol dehydrogenase.

Reagents

Proteolytic enzymes were as described by Shotton & Hartley (1973). Iodo[¹⁴C]acetic acid was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., mixed with unlabelled iodoacetic acid and recrystallized from chloroform to the specific radioactivity indicated. NAD⁺ (Boehringer Ltd., London W.5, U.K.; grade A) was used in kinetic assays.

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Methods

Large-scale cultures

Cultures of strain A (100 litres) were grown by the Microbiological Research Establishment, Porton, Wilts., U.K., for 12h at 37°C in medium containing casamino acids (Difco Laboratories, Detroit, Mich., U.S.A.) (20g/litre), Na_2HPO_4 (5.8g/litre), KH_2PO_4 (3.0g/litre) and MgSO_4 (0.12g/litre). The cells were stored as a frozen paste at -20°C.

Smaller cultures of strains 2A1 or 3A11 were grown in this laboratory in 10-litre fermentors (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) in a similar medium with the addition of 1.0g of NH_4Cl /litre under vigorous aeration at 37°C. The MgSO_4 was added as a 1M-solution autoclaved separately and 2ml of propylene glycol monolaurate (K & K Laboratories Inc., Plainview, N.J., U.S.A.) was added to prevent foaming. The fermentors were inoculated with 200ml of a culture grown separately for 10h in similar medium. Cells were harvested after 12h in a Sharples Super centrifuge and stored at -20°C.

Enzyme assay.

Ribitol dehydrogenase activity was assayed by following the production of NADH at 340nm in a Gilford 2000 recording spectrophotometer with a Unicam SP.500 monochromator. Originally, following the method of Wu *et al.* (1968), assay mixtures (3ml) contained 100mM-sodium phosphate, 50mM-ribitol, 0.83mM-NAD⁺ and enzyme (added last) at pH11.0, 28°C. However, a pH-activity profile (Fig. 1) showed that the activity at pH11 is very sensitive to small variations in pH, and the enzyme is relatively unstable at this high pH. Most assays

were therefore subsequently made in 50mM-ribitol, 0.83mM-NAD⁺ and 100mM-sodium phosphate, pH7.0. All assays reported here are expressed as 'pH7 units' which represent the formation of 1 μmol of NADH/min in this latter system. For comparison with previous work, 1 such 'pH7 unit' = 4.5 'pH11 units'.

Protein

Protein was measured by a modified Lowry procedure (Miller, 1959).

Enzyme purification

Unless otherwise stated all steps in the purification were carried out at 4°C in potassium phosphate buffers, pH7.0. Between 1 and 2kg of frozen cell paste was thawed into 0.1M buffer to give an approx. 75% (w/v) suspension. The cells were ruptured by two passes through a French pressure cell (American Instrument Co., Ltd., Silver Springs, Md., U.S.A.) at pressures between 60 and 100MPa. The extract was diluted with an equal volume of 0.1M buffer and centrifuged at 23000g for 30min to remove cell debris. The pellets were washed with 0.1M buffer and the combined supernatants were diluted with 0.1M buffer to give 10mg of protein/ml. Then 0.1 vol. of streptomycin sulphate (40mg/ml in 10mM buffer) was added dropwise and after stirring for 15min the precipitate was removed by centrifugation at 18000g for 15min.

The supernatant was slowly adjusted to 35% saturation with $(\text{NH}_4)_2\text{SO}_4$, stirred for 20min and centrifuged at 23000g for 30min. This supernatant was slowly adjusted to 55% saturation with $(\text{NH}_4)_2\text{SO}_4$ and stirred and centrifuged as described above. The precipitate (approx. 50g) was resuspended in 200ml of 10mM buffer and dialysed over 24h against three changes of 10 litres of 10mM buffer. Any residual precipitate was centrifuged and the conductivity and pH were adjusted to ionic strength 0.01M, pH7.0. The sample was applied to a column (35cm $\times$ 6cm diam.) of DEAE-cellulose (Whatman DE-52) in equilibrium with 10mM buffer, washed with 10mM buffer (4ml/min) until no more protein emerged and then eluted with 60mM buffer. Active fractions were pooled, concentrated by ultrafiltration, diluted to a final concentration of 10mM buffer and applied to a second DE-52 column (42cm $\times$ 3.5cm diam.) in equilibrium with 10mM buffer. This column was eluted at 1.6ml/min with 4 litres of a buffer gradient linear from 10 to 100mM-potassium phosphate, pH7.0. Active fractions which were eluted at a conductivity of approx. 4mmhos were pooled, concentrated by ultrafiltration and adjusted to 1mM-NAD⁺ and 60–80mg of protein/ml. This sample (15–20ml) was applied to a column (58cm $\times$ 3cm diam.) of Sephadex G-200

Fig. 1. pH-activity curve of ribitol dehydrogenase

Assays were done at 28°C in 50mM-ribitol-0.83mM-NAD⁺-0.1M-potassium phosphate (●) or carbonate (○) buffers at the indicated pH. Results are plotted as percentage of maximum activity.

(fine grade) equilibrated with 1mM-NAD⁺-10mM buffer and eluted by upward flow at 10ml/h. Active fractions (10ml) were pooled, concentrated by ultra-filtration to approx. 30mg of protein/ml and stored at -20°C.

Enzyme kinetics and inhibition

Assays were made at 28°C with samples of pure enzyme from strain A (0.3 µg) in 1.2ml of 0.83mM-NAD⁺, 0.1M-potassium phosphate, pH7 or pH11, and concentrations of ribitol between 0.4 and 83mM or of xylitol between 0.2 and 1.33M. $K_{m(app.)}$ and V_{max} were determined from reciprocal plots. The effect of various inhibitors was tested by preincubating the enzyme with the compound for 5min in 0.1M-potassium phosphate-5% (v/v) ethanol, pH7.0, at 25°C and then assaying samples at pH7 or 11. Protection by NAD⁺ was tested by preincubating the enzyme with 1mM-NAD⁺-0.1M-potassium phosphate, pH7.0, for 5min before addition of the inhibitor. In some cases, inhibition constants were determined by assaying at several inhibitor and ribitol concentrations.

Ultracentrifugation

Sedimentation equilibrium was carried out in a Beckman model E ultracentrifuge by using interference optics on solutions of ribitol dehydrogenase (1.55mg/ml) in 0.4mM-NAD⁺-0.1M-KCl-0.01M-potassium phosphate, pH7.0, at 4°C as described by Durham (1972) at a rotor speed (ω) of 1494 radians/s. A partial specific volume ($\bar{v}$) of 0.73 was assumed, and specific gravity (ρ) 1.004. The sedimentation velocity was measured for similar solutions at a rotor speed of 59780rev./min. Sedimentation-equilibrium studies were also made of enzyme (2mg/ml) in 5mM- β -mercaptoethanol-5M-guanidinium chloride-0.1M-potassium phosphate, pH7.0, at 5°C at a rotor speed (ω) of 2276 radians/s. By weighing known volumes the specific gravity (ρ) was determined to be 1.130 at 20°C and $\bar{v}$ was assumed to be 0.73.

Polyacrylamide-gel electrophoresis

Samples from the various purification steps were submitted to electrophoresis on 7% (w/v) polyacrylamide gels (Davis, 1964) and the gels were sliced in half longitudinally. One half was stained overnight with 1% (w/v) Amido Black in 7% (v/v) acetic acid, then destained electrophoretically and stored in 7% (v/v) acetic acid. The other half was stained for enzymic activity (Moore & Vilee, 1963) by submerging it in a solution containing 0.1M-ribitol, 0.8mM-NAD⁺, 0.3mg of Nitro Blue Tetrazolium/ml, 0.05mg of hydroxyphenazine methosulphate/ml and 10mM-Tris, pH11.0. When the purple band denoting

enzyme activity became visible the gels were washed with water and stored in 7% (v/v) acetic acid. To determine subunit size, samples were run in parallel with marker proteins of known subunit size in the presence of 0.1% (w/v) sodium dodecyl sulphate on 10 or 13% polyacrylamide gels with 3% stacking gels (Laemmli, 1970).

Amino acid analysis

Samples of protein were dialysed for 24h against water and freeze-dried. Samples were hydrolysed at 105°C in sealed evacuated tubes containing 6M-HCl saturated at room temperature with phenol. After evaporation to dryness, they were analysed in an automatic amino acid analyser.

A time-course of hydrolysis was determined for ribitol dehydrogenase from strain A on both native and performic acid-oxidized protein (Hirs, 1956). The experimental decay of serine and threonine allowed correction to zero time of hydrolysis. The values for valine and isoleucine rise to a maximum at 72-108h.

For enzyme from strain A611 samples of the oxidized or carboxymethylated chains that had been purified by ion-exchange chromatography were hydrolysed in parallel with chymotrypsinogen. The known composition of the latter allowed corrections to be made for destruction of other amino acids under the experimental conditions used. These amounted to less than 3% except for methionine (20%), tyrosine (10%), and lysine (9%).

Tryptophan was determined spectrophotometrically (Beaven & Holiday, 1952) on peptic digests of ¹⁴C-labelled ribitol dehydrogenase from strain A11. The protein (2.5mg) was incubated with pepsin (30 µg) in 4ml of 0.1M-HCl for 18h at 20°C, with parallel controls containing 1mg of chymotrypsinogen and pepsin or pepsin only. After addition of 1ml of 0.5M-NaOH, the extinction was measured at 280.0 and 294.4nm, giving a tyrosine/tryptophan ratio of 1.30 for ribitol dehydrogenase and 0.56 for chymotrypsinogen. Assuming a tyrosine/tryptophan ratio of 4:8 for chymotrypsinogen and a content of three tyrosine residues per subunit (mol.wt. 27000), this corresponds to 2.6 tryptophan residues per subunit.

Protein-chemical methods

Carboxymethylation was carried out on samples of enzyme (15mg/ml) that had been dialysed overnight at 4°C against a N₂-gassed buffer containing 0.1mM-EDTA, 2mM-dithiothreitol, 0.2M-Tris-HCl, 8M-urea, pH8.5. Iodo[¹⁴C]acetic acid was recrystallized from chloroform and diluted with carrier to specific radioactivity 0.20 µCi/µmol. A sample of this in 0.2M-Tris-HCl, pH8.5, was added to the denatured enzyme to give a final concentration of 10mM-iodo-

acetic acid. After 2 h at 20°C, β -mercaptoethanol was added to final concentration 20mM and the solution was dialysed against 10mM-NH₄HCO₃. In some cases, the [¹⁴C]carboxymethylated ribitol dehydrogenase was dialysed against 20mM-Tris-HCl-8M-urea, pH 8.0 and the sample (100–200mg in 20ml) was purified on a column (30cm×3.6cm diam.) of DEAE-cellulose (Whatman DE-52) with a linear gradient (1 litre) of 20mM–0.5M-Tris-HCl-8M-urea, pH 8.0, at 40ml/h.

Performic acid oxidation of the enzyme was done as described by Hirs (1956). Occasionally the oxidized ribitol dehydrogenase was purified by ion-exchange chromatography as described above.

Enzymic digests were carried out by using native, oxidized or carboxymethylated ribitol dehydrogenase (1–5mg/ml) and trypsin, chymotrypsin or thermolysin (10–50µg/ml) in 10mM-NH₄HCO₃ for 4 h at 37°C. Pepsin digests contained 5mg of ribitol dehydrogenase and 0.1 mg of pepsin in 1 ml of 5% (v/v) formic acid.

Other protein-chemical methods were as described by Shotton & Hartley (1973).

Results

Enzyme purification

Table 1 summarizes the purification procedure for 1 kg of strain 2A cells. The addition of 1mM-NAD⁺ to the fractions immediately after gradient elution from the DE-52 column appeared to stabilize the enzyme. Fig. 2 shows the elution profile from the final Sephadex G-200 column. Gel electrophoresis on the pooled fraction shown in Fig. 2 shows a single band of protein coincident with the activity stain (Plate 1). A final yield of between 300 and 600mg of pure enzyme was reproducibly obtained. Table 1 shows that ribitol dehydrogenase represents about 1.2% of the total soluble protein in this constitutive strain.

The evolution in our chemostat experiments (Hartley *et al.*, 1972) of strain 2A1, which produces large quantities of ribitol dehydrogenase allowed us to grow sufficient quantities of cells within this laboratory. Three 10-litre cultures of strain 2A1 yielded 129 g of cells. Table 1 summarizes the purification of the enzyme from these cells and Plate 2 shows polyacrylamide gels at various stages during the purification. The enzyme is clearly one of the two major proteins in the extract. Neither is found at this intensity in extracts of wild-type cells. It was essentially pure after gradient elution from the DE-52 column, and a final yield of 340mg was obtained. The enzyme is about 6% of the total soluble protein in the extract.

Table 1 summarizes the purification from 147 g of strain A11 cells from 3×10 litres of culture. The specific activity and gel electrophoresis showed that the

Fig. 2. Final step in the purification of ribitol dehydrogenase. Elution profile from Sephadex G-200 (58 cm×3 cm diam.): ●, E_{280} ; ○, ribitol dehydrogenase activity (5µl samples in 3ml of pH 11 assay mixture).

Table 1. Purification of ribitol dehydrogenase from 'ancestral' and 'evolant' strains

The values for total enzyme units are normalized to a preparation from 1 kg of cells.

Purification step	Strain ...	10 ⁻³ × Total enzyme activity (units)			Specific activity (units/mg of protein)			Purification			Yield (%)		
		2A	2A1	A11	2A	2A1	A11	2A	2A1	A11	2A	2A1	A11
Crude extract		180	880	2750	1.0	5.1	18.7	1	1	1	100	100	100
Streptomycin supernatant		160	600	1700	—	—	—	—	—	—	92	69	62
35–55%-satd. (NH ₄) ₂ SO ₄ precipitate		100	450	1100	2.3	18.8	36.5	2.2	3.6	1.9	59	52	39
Batch DE-52 60mm eluate		75	400	800	10.3	74	119	10.0	14.4	6.4	44	46	29
Gradient DE-52 eluate		70	240	450	55	87	107	52	16.9	5.8	38	27	16
Sephadex G-200 eluate		45			83			80			26		

EXPLANATION OF PLATE I

Final step in the purification of ribitol dehydrogenase from strain 2A

Polyacrylamide gels of samples of the pooled fractions from the Sephadex G-200 step (horizontal arrows in Fig. 2) stained for (a) protein; (b) ribitol dehydrogenase activity.

EXPLANATION OF PLATE 2

Steps in the purification of ribitol dehydrogenase from strain 2A1

Polyacrylamide gels of samples taken at each step, stained for protein unless otherwise stated. (a) Crude extract; (b) streptomycin supernatant; (c) 35% (w/v)-satd. $(\text{NH}_4)_2\text{SO}_4$ supernatant; (d) 35-55% (w/v)-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate; (e) as (d) stained for ribitol dehydrogenase activity; (f) 60mm buffer eluate from DE-52 batch step; (g) pooled active fractions from DE-52 gradient step.

enzyme was essentially pure after the batch elution from DE-52, though gradient elution was necessary to remove a slight contaminant. The enzyme was 18% of the cell extract and the final yield is 3–6g/kg of cells. Gels, run at earlier stages of the purification, again showed two heavily staining protein bands of roughly equal intensity. It appears that the synthesis of a second protein has also been increased by the mutations that lead from strain 2A to 2A1 and from strain 2A1 to A11.

Stability, activity and inhibition

Enzyme purified as above can be stored in 1mM-NAD⁺–10mM-sodium phosphate buffer, pH7.0, at –20°C, for several months with only slight loss in activity. After 6 months one preparation which retained 75% of its original activity was incubated overnight in 0.1mM-dithiothreitol at 4°C and fractionated on Sephadex G-200 in 0.1M-sodium phosphate–0.1mM-NAD⁺–0.1mM-dithiothreitol, pH7. The major peak (about 80% of the total protein) had 98% of the original specific activity.

However, subsequent studies (B. D. Burleigh, P. J. W. Rigby & B. S. Hartley, unpublished work) showed that prolonged storage of some enzyme preparations generates three closely spaced bands on gel electrophoresis, each of which stains for ribitol dehydrogenase activity (see Plate 2g). It is probable that this is due to minor proteolytic modification of the native enzyme, since it is prevented by storage in the presence of 0.1mM-phenylmethanesulphonyl fluoride. For this reason 0.1mM-phenylmethanesulphonyl fluoride has been included in all buffers during recent purifications of ribitol dehydrogenase.

Preliminary kinetic observations were made both on crude extracts and on the purified enzyme from strain A. In the pH11 assay, $K_{m(app.)}$ for ribitol was 5mM, for xylitol 1.0M and for NAD⁺ 0.1mM. The ratio $K_{m(app.)}$ for ribitol/ $K_{m(app.)}$ for xylitol was 0.005 and the ratio V_{max} for ribitol/ V_{max} for xylitol was 5.0. These ratios were constant for crude extracts and for enzyme at various steps in the purification.

The enzyme is unstable in dilute solutions; NAD⁺ (1mM) protects the activity for several days at 2°C, but 5mM-nicotinamide, 1mM-dithiothreitol or 50mM-ribitol have no stabilizing effect. Incubation with 50mM-*o*-phenanthroline or 50mM-diethyldithiocarbamate gives over 50% inhibition, but NAD⁺ protects against this. There is no reactivation by added metal ions. EDTA has no effect.

Heavy metals inhibit, e.g. mercuric chloride (K_i 16μM) or *p*-chloromercuribenzoate (K_i 20mM), but NAD⁺ protects. Dithiothreitol (K_i 25mM) and β-mercaptoethanol (K_i 220mM) appear to be competitive with ribitol. GSH has no detectable effect on the activity.

Molecular weight and subunits

Samples (4–5ml) of native enzyme from strain 2A and protein markers were run on a Sephadex G-200 column (45cm×3cm diam.) eluted by upward flow (10.6ml/h) with 1mM-NAD⁺–0.1M-potassium phosphate, pH7.0, 5ml fractions being collected. Blue Dextran and NaCl were used to determine void volume (V_0) and total volume (V_t) respectively. The elution volumes (V_e) of yeast alcohol dehydrogenase (mol.wt. 150000), pig glyceraldehyde phosphate dehydrogenase (mol.wt. 144000) and ribitol dehydrogenase were determined by appropriate spectrophotometric assays of enzyme activity. Bovine serum albumin was determined by its extinction at 280nm. Fig. 3 shows the partition coefficient, $K_{av} = (V_e - V_0)/(V_t - V_0)$ plotted logarithmically against molecular weight, giving a molecular weight for the enzyme of 100000–110000.

Sedimentation equilibrium of the native enzyme at 4°C in the presence of 1mM-NAD⁺ at pH7 gave a linear slope of $d(\log c)/dr^2$ (Fig. 4a), indicating a homogeneous species of molecular weight 118000 ± 2000 assuming $\bar{v}$ is 0.73 ± 0.01 . The sedimentation coefficient under similar conditions was 6.04S. Sedimentation equilibrium in the presence of 5M-guanidinium chloride (Fig. 4b) showed a single species of molecular weight 28900 ± 2000 assuming $\bar{v}$ is 0.73 ± 0.01 . Polyacrylamide gels run in the presence of sodium dodecyl sulphate with marker proteins of known molecular weight gave a single band for ribitol dehydrogenase from strain 2A, corresponding to a molecular weight of 25000 (Fig. 5). Previous determinations of the molecular weight of this enzyme gave values of 140000 by Archibald's method (Fromm & Bietz, 1966) or 99000 by sedimentation equilibrium (Fromm &

Fig. 3. Molecular weight of native ribitol dehydrogenase by gel filtration

Samples of enzyme from strain 2A ribitol dehydrogenase (RDH), yeast alcohol dehydrogenase (YADH), pig glyceraldehyde phosphate dehydrogenase (GPDH), bovine serum albumin (BSA), Blue Dextran and NaCl were run on a Sephadex G-200 column as described in the text, and partition coefficients (K_{av}) were calculated from the elution volumes.

Fig. 4. Sedimentation equilibrium of ribitol dehydrogenase

(a) Native enzyme from strain 2A (1.55 mg/ml) in 0.4 M-NAD⁺-0.1 M-KCl-0.01 M-potassium phosphate, pH 7.0, at 4°C was sedimented at 1494 radian/s. The slope (1.54) gives molecular weight (*M*) 118 000 calculated from the equation

$$M = \frac{d \ln c}{dr^2} \times \frac{2RT}{\omega^2(1-\bar{v}\rho)}$$

(Durham, 1972) by using $\bar{v} = 0.73$ and $\rho = 1.004$. (b) Enzyme from strain 2A (2.0 mg/ml) in 5 mM- β -mercaptoethanol-5 M-guanidinium chloride-0.1 M-potassium phosphate, pH 7.0, at 5°C was sedimented at 2276 radian/s. The slope (0.565) gives molecular weight 28 900.

Bietz, 1966). Mortlock *et al.* (1965) reported a molecular weight of 110 000 by sucrose gradient centrifugation. Our own preliminary studies (Hartley *et al.*, 1972) suggested a tetramer of $4 \times 32 000$, but we have discovered calculation errors in some of these data and they now agree with the above results. Taken in conjunction with the amino acid analysis and peptide 'maps' which will be described next, the data point to a tetramer of four identical subunits each of molecular weight 27 000.

Fig. 5. Polyacrylamide-gel electrophoresis of ribitol dehydrogenase in the presence of sodium dodecyl sulphate

10% (broken line) or 13% (solid line) cross-linked gels were run in the presence of 0.1% sodium dodecyl sulphate by the method of Laemmli (1970). Protein markers: *Escherichia coli* β -galactosidase, GA; bovine serum albumin, BSA; human wart virus coat protein, WV; ovalbumin, OV; rabbit creatine phosphokinase, CPK; pig glyceraldehyde phosphate dehydrogenase, GPDH; rabbit triose phosphate isomerase, TIM; bovine chymotrypsinogen-A, CG; ribitol dehydrogenase, RDH; tobacco mosaic virus protein, TMV.

Amino acid composition and peptide 'maps'

Table 2 shows the composition of various samples of ribitol dehydrogenase from some of the 'evolvants' described by Hartley *et al.* (1972), assuming identical subunits of mol.wt. 27 000. The small analytical differences between different preparations are within experimental error, and there is no reason to believe that the compositions of the enzymes from each of these strains are other than identical.

To confirm the size and establish the identity of the subunits, the enzyme was treated with iodo[¹⁴C] acetic acid, digested with trypsin and submitted to two-dimensional paper electrophoresis and chromatography. The 'maps' were examined by radioautography and stained with either ninhydrin or specific stains for arginine, histidine, tyrosine and tryptophan. The results (e.g. Fig. 6) showed 26 well-resolved spots compared with the 22 expected from the amino acid analysis. However, some of these were present in low yield and probably arise from incomplete tryptic cleavage or from chymotrypsin contamination. Moreover, there are a number of peptides that remain stuck to the origin during electrophoresis and trail badly during chromatography.

A methionine diagonal peptide 'map' (Tang & Hartley, 1967) of tryptic digests of carboxymethylated enzyme revealed three basic peptides which lay off the diagonal. To identify these peptides, a sample of

Table 2. *Amino acid analysis of ribitol dehydrogenase from various strains*

Samples of native (Nat.), performic acid-oxidized (Ox.) or carboxymethylated (CM.) enzymes were hydrolysed as described in the Methods section. Cysteine was determined as cysteic acid or carboxymethylcysteine and methionine as methionine sulphone where appropriate. Results for threonine and serine are corrected to zero hydrolysis time; those for valine and isoleucine are the maximum values obtained in each set of hydrolyses. The results for strain A611 are for the chains purified after oxidation and carboxymethylation and are corrected by comparison with parallel hydrolysates of chymotrypsinogen. Tryptophan was determined spectrophotometrically (Beaven & Holiday, 1952) on peptic digests of strain A11 with parallel controls of chymotrypsinogen.

Composition (residues/subunit)										
Amino acid	Strain ...	A			A11	A21	A211	A611		Best value
		Nat.	Ox.	CM.	Nat.	Nat.	Nat.	Ox.	CM.	
Cys	—	2.0	1.9	—	—	—	1.6	—	2	
Asp	23.8	24.2	28.0	23.7	23.5	23.7	25.4	24.2	24	
Thr	11.7	12.3	11.2	11.4	10.4	10.1	11.2	10.9	11	
Ser	15.7	15.7	14.5	13.4	14.8	13.2	15.2	14.8	15	
Glu	21.0	21.0	21.4	22.8	21.0	19.8	21.2	19.9	21	
Pro	9.7	9.8	8.6	10.7	9.5	10.8	10.5	10.2	10	
Gly	20.2	19.9	20.0	20.0	20.6	20.0	19.8	19.3	20	
Ala	29.0	27.7	26.6	29.4	28.6	27.7	30.0	29.2	29	
Val	27.8	28.0	28.0	28.8	30.6	28.9	33.3	33.2	33	
Met	5.2	4.6	4.1	5.7	6.0	5.3	5.9	5.9	6	
Ile	13.8	13.7	13.7	14.7	13.6	13.3	15.3	15.5	15	
Leu	26.9	25.8	24.4	27.1	24.8	24.6	25.8	28.0	27	
Tyr	3.4	3.4	3.1	3.2	2.9	3.0	3.0	3.1	3	
Phe	6.2	6.2	6.4	6.1	7.0	6.7	7.0	7.0	7	
Trp	—	—	—	2.6	—	—	—	—	3	
Lys	9.6	9.2	9.9	9.0	7.1	9.1	9.2	9.4	10	
His	5.2	5.1	4.9	4.2	4.7	5.1	4.9	4.9	5	
Arg	10.8	9.8	10.8	9.2	10.7	10.4	10.8	10.9	11	
Total									252	
Mean of:	3	3	1	2	1	3	4	4		
Hydrolysis time (h)	12, 31 and 48			108	48	24	24	12, 36, 72 and 108		

Table 3. *Peptide 'maps' of various digests of ribitol dehydrogenase*

The number of major spots and minor spots (in parentheses) revealed by appropriate reactions in peptide 'maps' of various enzymic digests of carboxymethylated, oxidized or native enzyme is compared with the number expected for identical subunits of mol.wt. 27000 based on the amino acid analysis (Table 2).

Peptides containing:	Trypsin*	Trypsin+ chymotrypsin	Pepsin	Thermolysin	Expected
Arg	7 (2)	12 (3)	—	13 (1)	11
His	3 (1)	—	6 (2)	3 (3)	5
Tyr	1 (0)	2 (1)	3 (0)	—	3
Trp	1 (0)	3 (0)	3 (1)	3 (1)	3
CmCys	2 (0)	2 (0)	—	—	2
Met	2 (1)	—	—	—	6
Total	17 (9)	35-40	70-80	45-50	22†

* Plus insoluble 'core'.

† Number of tryptic peptides expected.

carboxymethylated enzyme was incubated with 0.1 M-iodocetamide in 0.2 M-pyridine acetate, pH 3.5, for 5 h at 37°C. The product, which should contain carboxymethylcysteine and carboxamidomethylmethio-

nine, was dialysed, digested with trypsin and 'mapped' as in Fig. 6. The absence of the three spots marked in Fig. 6 showed that they originally contained methionine. Table 3 summarizes the results from

Fig. 6. Peptide 'map' of a tryptic digest of [^{14}C]carboxymethylated ribitol dehydrogenase

Major spots staining with ninhydrin are in black; minor components are hatched. The results of colour reactions for specific amino acids or of radioautography are indicated. *m* indicates electrophoretic mobility in pyridine acetate at pH 6.5 relative to Asp = -1.0. *n* indicates electrophoretic mobility in formic acid at pH 2 relative to Ser = 1.0. *R* indicates chromatographic mobility in butan-1-ol-acetic acid-water-pyridine (15:3:12:10, v/v) (BAWP) relative to 1-dimethylaminonaphthalene-5-sulphonic acid.

peptide 'maps' of other enzymic digests of ribitol dehydrogenase. The tryptic 'maps' are ambiguous because of the insoluble 'core', but all the other digests were completely soluble and indicate that the four subunits are identical.

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