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Properties of three isoenzymes of *Clostridium pasteurianum* hydrogenase

ACKRELL, ASATO AND MOWER[1,2] showed the existence of a maximum of six hydrogenase (H_2 ferredoxin oxidoreductase, EC 1.12.1.1) isoenzymes in *Clostridium pasteurianum*. Techniques such as gel filtration and density gradient centrifugation allow the estimation of molecular size of enzymes in the presence of other proteins. They are thus particularly useful in the study of hydrogenase enzymes which, because of their well known instability[3,4], have not been purified. We report here the molecular weight and the interrelation of three of the most active hydrogenase isoenzymes (α, β, and γ).

C pasteurianum cells were grown in the medium described by CARNAHAN AND CASTLE[5]. Extracts were obtained by autolysis of 1.0 g of dried cells in 10 ml of 0.1 M potassium phosphate buffer (pH 7.0)[6], followed by centrifugation at 30 000 × *g* for 30 min. The hydrogenase-containing supernatant was stored under hydrogen.

The presence of hydrogenase activity in different bacterial extracts was determined quantitatively by manometric evolution of H_2 gas according to the method of PECK AND GEST[7]. Individual hydrogenase isoenzyme species were identified by their R_F values after hydrogenase preparations (1–5 mg of protein) were subjected to electrophoresis on polyacrylamide disc gels (7.5%)[8]. Bromophenol blue was used as the zone front for R_F determinations. The site of hydrogenase activity in the gel was located by the method of ACKRELL, ASATO AND MOWER[1] in which methyl viologen is reduced in the presence of H_2 gas.

The vertical slab gel apparatus of RAYMOND[9] was used with the same buffer

and gel system as described by ORNSTEIN AND DAVIS[8] to isolate single hydrogenase species After a sample of crude extract (20–30 mg protein) had been subjected to electrophoresis, the appropriate section of the gel was excised, and homogenized in 2–3 vol of 0 1 M phosphate buffer (pH 7 0) kept reduced with 0.1% sodium dithionite After the homogenate had been centrifuged at 30 000 × *g* for 20 min, an aliquot of the supernatant containing the appropriate hydrogenase isoenzyme was immediately rerun on a polyacrylamide disc gel to confirm its identity and the remainder used for subsequent experimentation.

Disc gel columns as described above with the concentration of acrylamide varied from 6 to 14% were used in experiments comparing the molecular weights of enzymatic forms[13]

Sucrose density gradient centrifugation was performed as described by MARTIN AND AMES[10] using 5–20% sucrose in 0 1 M phosphate buffer (pH 7 0) Sodium dithionite (0 25%) with methyl viologen as redox indicator was incorporated in the gradient to ensure anaerobic conditions throughout the experiment Fractions were collected for polyacrylamide gel assay or manometric assay Beef heart cytochrome *c* and rabbit hemoglobin were used as standards in separate centrifuge tubes

C pasteurianum preparations were passed through columns of Sephadex G-100 (1 6 cm × 113 cm) and G-75 (1 6 cm × 100 cm), prepared and eluted according to the method of ANDREWS[11] except that the molarity of the Tris-HCl buffer (pH 7 5) was 0.2 M and it was kept reduced with 0 05% sodium dithionite *plus* methyl viologen as redox indicator The columns were standardized for molecular weight estimation by determining the elution pattern of each of the following. blue dextran, beef heart lactate dehydrogenase, bovine serum albumin, trypsin and bovine heart cytochrome *c*. The compounds were detected by absorbance at 280 mμ.

A small amount of solid sodium dithionite added to each collection tube ensured anaerobic conditions after elution

The three most active hydrogenase forms of *C pasteurianum* were studied and these had R_F values on 7 5% polyacrylamide gel of 0 75 ±0 05, 0 64 ±0 05, and 0.55 ±0.05 and were therefore designated as the α, β and γ forms, respectively The highest activity could be attributed to the γ species since the band of reduced methyl viologen produced by it invariably appeared first and spread most rapidly within the gels Individual species obtained from the RAYMOND gel slab and identified as such by immediate assay on polyacrylamide disc gels were stored (4°) overnight at pH 7 0 and pH 10.0. The α and β species were entirely converted to the γ species, 0 7 M, 1 5 M β-mercaptoethanol did not affect this conversion Incubation with 3 M β-mercaptoethanol, 1 M NaCl, 8 M urea, 1 M guanidine HCl resulted in elimination of all activity by the gel assay

Sedimentation of crude preparations in sucrose gradients resulted in a 95% loss in activity The presence of a particular hydrogenase species in a fraction from the gradient was determined by the disc electrophoresis technique The peak of activity for a particular hydrogenase isoenzyme was assumed to occur in the middle of a consecutive series of fractions which contained that hydrogenase isoenzyme If sufficient activity allowed, the manometric assay was used to confirm the peak activity The specific activity of the hydrogenase was increased 100% by using 5 times the amount of iron prescribed by CARNAHAN AND CASTLE[5] in the growth medium This was reflected in the greater activity accorded both the α and γ hydrogenase species from

TABLE I

SEDIMENTATION STUDIES ON THE ISOENZYMES

Sedimentation coefficients were determined by sucrose gradient centrifugation The S E is quoted for γ

Isoenzyme	*Reference protein*	*Mean* $s_{20,w} \times 10^{13}$	*Estimated mol wt*	*Number of times isoenzyme activity retained per number of determinations*	
				With normal cells	*With high iron cells***
γ	Hb	4 0 ± 0 35	56 000 ± 7000	8/8	2/2
β*	Hb	3 8	51 000	2/8	0/2
α*	Hb	4 2	59 000	0/8	2/2

* These always appeared in conjunction with the γ form

** $FeSO_4$ concn (250 mg/l) was 5 times that recommended[5] in the original medium

cells grown in this iron-enriched medium when analyzed by the polyacrylamide gel technique. The reduced methyl viologen produced by such a γ band within a disc gel spread so rapidly that it generally obscured that produced by the β band. The results of the sedimentation analyses are shown in Table I

The $s_{20,w}$ value of the predominant hydrogenase form, the γ species, was 4 0 S When the position of the more labile α and β species could be located, they also had an s value that fell within the 95% confidence range of the most stable form Provided the molecule is assumed to be spherical and of partial specific volume, 0 725, the molecular weights of the α, β, γ species estimated from the s value[10,12] are the same at 56 000 ±7000.

In all 15 gel filtration experiments a single peak of hydrogenase activity was eluted and as expected, the analysis of fractions by disc electrophoresis established the presence of each species (α, β and γ) at the same elution volume The average molecular weights of the three species (shown in Table II) were not significantly different, thus confirming the results from the sedimentation studies.

TABLE II

MOLECULAR WEIGHTS OF THE ISOENZYMES FROM GEL FILTRATION

The S E is quoted for γ and the number of determinations is shown in parentheses.

Isoenzyme	*Number of runs yielding the active form*	*Mean mol wt*	*$FeSO_4$ concn (mg/l)*
γ	14	53 000 ± 5000	50** (12)
			250 (2)
β*	6	54 000	50 (6)
α*	4	50 500	50 (2)
			250 (2)

* These always appeared in conjunction with the γ form

** Refers to the amount of $FeSO_4$ recommended in the original medium[5]

Preparations of the isolated hydrogenase species were subjected to disc electrophoresis at polyacrylamide concentrations varying between 6% and 14%. The ratios of the R_F values of the α and β bands to that of the γ band remained constant This suggested again that the three species possessed the same molecular size but differed in resultant charge.

Thus we have shown that the three hydrogenase isoenzymes, the α, β and γ species, were interrelated with an equilibrium in favor of the more stable γ species and their respective sizes would appear to be similar with a molecular weight of 50 000–60 000

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