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Comparative studies of bacterial hydrogenase

Comparative studies of the physical properties of hydrogenase (H_2:ferredoxin oxidoreductase, EC 1.12.1.1) enzymes have been virtually precluded because of their general instability and sensitivity to oxygen. Recently, we have studied[1] these enzymes by using acrylamide-gel electrophoresis, density-gradient centrifugation and gel filtration. These procedures do not require prior purification of the enzyme under study, and their application to the hydrogenase enzymes has shown that most of these enzymes are extracted as mixtures of several distinct molecular species[2]. The three main forms of hydrogenase from *Clostridium pasteurianum* have been shown to be of the same molecular weight.

We wish to report in this communication an extension of this work to the measurement of the size and shape of hydrogenase enzymes from a variety of representative microorganisms.

The sources and culture conditions of the microorganisms have been reported[2]. The cells were harvested in the log phase of growth. The extracts were obtained either by autolysis of dried cells according to MORTENSON[3] or by sonic treatment of cell pastes. All nonsedimentable material at 144 000 $\times g$ for 30 min was deemed soluble in this investigation. One preparation of *Clostridium butylicum* hydrogenase was purified 400-fold by a modification of the procedure of PECK AND GEST[4]. The method of KONDO *et al.*[5] was used to solubilize membrane-bound hydrogenases.

Hydrogenase was detected in fractions after gel filtration or sucrose density-gradient centrifugation experiments either by a quantitative manometric assay using the redox dye methyl viologen to catalyze H_2 evolution[6], or methylene blue for H_2 uptake. Alternatively, a more sensitive qualitative assay using acrylamide gels has been reported[1,2]. In this latter assay, the location of the maximum hydrogenase activity in column eluates was taken as the central fraction of a consecutive series of fractions, all showing hydrogenase activity on the acrylamide gels. Comparison of the two assays in the same experiment showed the activity peak to be present in the same fraction. The presence of aggregate material possessing hydrogenase activity was deduced from the appearance of hydrogenase activity at the top of the acrylamide-gel column, indicating the presence of material of molecular weight greater than $1 \cdot 10^6$ which could not enter the gel.

Sucrose density-gradient centrifugation was carried out as previously described[1,8]. Bovine-heart cytochrome *c*, rabbit Hb, bovine-liver catalase, rat-liver ribosomes and feritin were used as standards. Gel columns using Sephadex G-75, G-100, G-150, G-200 and Bio-Gel A5 were prepared using the method of ANDREWS[7]. The columns were calibrated for molecular weight and Stokes radius by determining the elution volume of the following standards: bovine thyroglobulin, fibrinogen, bovine-heart lactate dehydrogenase, apoferitin, α-globulin, bovine serum albumin, ovalbumin, chymotrypsinogen A (bovine pancreas), trypsin and cytochrome *c*. These protein standards were obtained from commercial sources and were used without further purification. Blue dextran 2000 was used to determine the void volume of the Sephadex gels. *Escherichia coli* was used for this purpose in the Bio-Gel A5 column. The molecular weights of the hydrogenase enzymes were initially estimated from

density-gradient sedimentation analysis by assuming the proteins to be spherical and of partial specific volume 0.725 (refs. 8 and 9). An improved procedure was used which did not require the first assumption and which enabled the frictional ratio (f/f_0) to be estimated in addition to the molecular weight. In this procedure, the gel filtration columns were calibrated according to the Stokes radii of standard proteins. The value of the Stokes radius for each hydrogenase enzyme was then used in combination with the corresponding sedimentation coefficient to obtain the molecular weight and f/f_0. The equations of SIEGEL AND MONTY[10] and of LAURENT AND KILLANDER[11] were used in these calculations.

Isoelectric point determinations were carried out using acrylamide gels of different pH values. The pH of the gel in the ORNSTEIN AND DAVIS[12] formulation was 9.5; the buffer system of WILLIAMS AND REISFELD[13] was used to obtain a pH of 8.0. Continuous buffer systems were employed to obtain pH's 7.0 and 6.0[14,15]. The isoelectric points of the hydrogenase enzymes were obtained by plotting pH *versus* R_F of the active species in the gel and by extrapolating to zero mobility.

In Table I are shown molecular weights and frictional ratios of a series of hydrogenase enzymes together with the distribution of activity between three identifiable forms of the enzyme sedimenting quite differently: (i) the free or unassociated forms, (ii) the aggregate, (iii) the particulate or membrane bound preparation that does not occur in the previously defined soluble fraction. Table I is divided into three sections; grouping organisms according to common metabolic patterns as suggested by GREY AND GEST[16]. Group I, strict anaerobes, contained the clostridia which have hydrogenases of similar molecular weight (approx. 55 000) except for *C. butylicum* hydro-

TABLE I

SIZE AND SHAPE OF HYDROGENASE ENZYMES FROM VARIOUS SOURCES

The values are for the unassociated or free form of the soluble enzyme. The number of determinations is shown in parentheses and the S.D. is quoted for *C. pasteurianum*; for the other species, the maximum deviation of each determination from the mean values reported never exceeded 10%.

Group	*Bacterial source*	*Mean* $s_{20,w} \times 10^{13}$	*Mean Stokes radius* $\times 10^8$ *(cm)*	*Mol. wt.* $\times 10^{-4}$	*Frictional ratio* (f/f_0)	*% Total activity* as soluble*	*% Soluble activity as aggregate after gel filtration*
I	*C. pasteurianum*	4.0 ± 0.35 (10)	30 ± 3 (14)	5.0	1.2	100	0
	C. felsineum	4.3 (2)	33 (2)	5.9	1.3	100	0
	C. butyricum	3.9 (2)	33 (2)	5.3	1.3	100	0
	C. butylicum	5.8 (3)	44 (3)	10	1.4	100	0
	C. butylicum (400-fold purified)	5.8 (2)	44 (2)	10	1.4	100	0
II	*P. vulgaris*	8.4 (3)	53 (3)	18	1.4	40	80
	E. coli	9.7 (3)	54 (3)	21	1.3	10	70
III	*D. desulfuricans*	4.6 (2)	30 (3)	5.6	1.1	20	5
	*A. vinelandii***	6.5 (2)	48 (2)	13	1.3	20	90

* This refers to the nonsedimentable hydrogenase remaining in solution after cellular disruption and centrifugation at 144 000 × *g* for 30 min.

** A strict aerobe not classified by GREY AND GEST[16].

genase which appears to have a molecular weight about twice that of the other clostridia and may represent a case of gene doubling[17]. These bacteria did not contain a membrane-bound hydrogenase, and the enzyme preparations did not form aggregates.

Group II contained facultative anaerobes. The soluble component of these preparations was resolved into an aggregate and unassociated form. The unassociated form had a much higher molecular weight than that of Group I (approx. 200 000). However, after gel filtration, most of the activity was recovered in the aggregate form which could represent self-association of the enzymes or their association with other cell components present in the extract.

The characteristics of the hydrogenase of *Desulfovibrio desulfuricans* were intermediate between Groups I and II. The soluble preparation contained hydrogenase which existed mainly as an enzyme of mol. wt. 56 000. After gel filtration, 95% of the recovered activity occurred in this form. However, a small amount of the enzyme was just resolved by the Bio-Gel A5 (exclusion mol. wt. $5 \cdot 10^6$), indicating the formation of some aggregate enzyme.

The soluble hydrogenase enzymes of each microorganism studied were composed of several distinct molecular species[2]. These species were not separated in our experiments and often several were recovered in the single hydrogenase activity peak. The finding that the three main hydrogenase species of *C. pasteurianum* were of the same molecular weight[1] may then hold true for many of the hydrogenase forms of other organisms as well. The frictional ratio results indicated that all the hydrogenases examined were approximately spherical proteins.

In the case of *A. vinelandii*, it is possible to solubilize a major portion of the membrane-bound enzyme. The sedimentation coefficients and isoelectric points of the unassociated soluble and solubilized particulate hydrogenase were compared (Table II) and found to be similar. The aggregate form contained most of the enzymatic activity, as found for the facultative anaerobes, and is apparently more stable than the unassociated material. The *A. vinelandii* aggregate can be separated into two particles of 51 and 77 S.

Although the results of these studies have been obtained with crude enzyme preparations, identical molecular weight values were obtained with an unpurified and 400-fold purified preparation of *C. butylicum*.

TABLE II

PHYSICAL PROPERTIES OF *A. vinelandii* HYDROGENASE

The unassociated form of the soluble and solubilized particulate preparations was characterized. The number of determinations is shown in parenthesis and the maximum deviation of each determination from the mean never exceeded 10%. The isoelectric points were obtained graphically and the R_F's by direct assay on acrylamide disc gels[1,2].

	R_F of Species	*$s_{20,w} \times 10^{13}$*	*Isoelectric point*	*$s_{20,w} \times 10^{13}$ aggregate*
A. vinelandii (soluble)	0.42, 0.47	6.5 (2)	5	
A. vinelandii (solubilized)	0.46	6.7 (3)	5	51, 77 (5)

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Department of Biochemistry and Biophysics,
School of Medicine, University of Hawaii,
Honolulu, Hawaii 96822 (U.S.A.)

A. D. KIDMAN*
R. YANAGIHARA
R. N. ASATO

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* Present address: Laboratory of Preclinical Pharmacology, National Institute of Mental Health, Saint Elizabeths Hospital, Washington, D.C. 20032, U.S.A.

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The subunits of porcine heart TPN-linked isocitrate dehydrogenase

The molecular weight of porcine heart TPN-linked isocitrate dehydrogenase (*threo*-D_s-isocitrate:TPN oxidoreductase (decarboxylating) EC 1.1.1.42) has been measured by MOYLE AND DIXON[1] and was found to be 64 000. SEIBERT *et al.*[2] computed a molecular weight of 61 000 from sedimentation and diffusion studies. Those authors obtained a sedimentation coefficient of 4.6 S and a diffusion coefficient of $7.3 \cdot 10^{-7}$ cm^2/sec^{-1}. To compute the above molecular weight, the authors assumed a partial specific volume of 0.75 ml/g. According to SEIBERT *et al.*[2], their preparation had a specific activity 8 times higher than that of MOYLE AND DIXON. The preparation of SEIBERT *et al.* was purified further by COLEMAN[3], and she obtained a molecular weight of 58 000. The fact that this enzyme exhibits conformational changes on addition of folate[4] and the fact that it displays two coenzyme binding sites as shown from fluo-