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In Vivo Inactivation of Glycerol Dehydrogenase in *Klebsiella aerogenes*: Properties of Active and Inactivated Proteins

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Glycerol:oxidized nicotinamide adenine dinucleotide (NAD^+) 2-oxidoreductase (EC 1.1.1.6), an inducible enzyme for anaerobic glycerol catabolism in *Klebsiella aerogenes*, was purified and found to have a molecular weight of 79,000 by gel electrophoresis. The protein seemed to be enzymatically active either as a dimer of a 40,000-dalton peptide at pH 8.6 or as a tetramer of 160,000 molecular weight at pH 7.0. The enzyme activity was present at high levels in cells growing anaerobically on glycerol, but disappeared with a half-life of about 45 min if molecular oxygen was introduced to the culture. In contrast, no such phenomenon occurred with dihydroxyacetone kinase activity, the second enzyme in the pathway. Immunochemical analysis showed that the inactivation of the oxidoreductase did not involve degradation of the protein. Furthermore, subunits of the active and inactive forms of the enzyme were indistinguishable in size on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and had similar isoelectric points (pH 4.7). Inactivation did, however, alter the gel filtration properties of the enzyme protein and, more importantly, reduced its affinity for the dye Cibacron F3GA and the coenzyme NAD^+.

Irreversible inactivation of specific biosynthetic and catabolic enzymes has been reported in a variety of eukaryotic and prokaryotic organisms (6, 8, 14, 15, 20, 23, 31). In vivo degradation of enzymes and modification to less active forms are believed to be important mechanisms by which the activities of competing metabolic pathways can be regulated, and wasteful cycling of metabolites through unproductive or harmful pathways can be promptly halted (23). Examples of enzyme inactivation in sporulating bacteria (27) and yeast (2) also suggest that such reactions may be important in morphogenesis. Although a variety of enzymes in microorganisms are known to be subject to in vivo inactivation, in only a few cases have the mechanisms involved been characterized. These include the phosphorylation of pyruvate dehydrogenase in *Neurospora crassa* (30), the irreversible dissociation of glutamine synthetase induced by NH_4^+ ions in *Candida utilis* (19), the adenylylation of glutamine synthetase in *Escherichia coli* (21), the deacylation of citrate lyase in *Rhodopseudomonas gelatinosa* (5), the oxidation of an iron-sulfur center of glutamine phosphoribosylpyrophosphate amidotransferase in *Bacillus subtilis* (26), and the proteolysis of uridine nucleosidase in yeast (11).

Glycerol dehydrogenase (EC 1.1.1.6; glycerol: NAD^+ 2-oxidoreductase) of *Klebsiella aerogenes*, hereafter also referred to as the dehydrogenase, is an example of a catabolic enzyme with activity that is irreversibly lost under aerobic conditions (9). This enzyme catalyzes the first step of the anaerobic pathway for growth on glycerol. The product, dihydroxyacetone, is phosphorylated by an ATP-dependent dihydroxyacetone kinase to form dihydroxyacetone phosphate.

When the cells are grown aerobically on glycerol as carbon source, levels of the dehydrogenase and dihydroxyacetone kinase are insignificant. Instead, glycerol is converted to *sn*-glycerol 3-phosphate, which is then oxidized to dihydroxyacetone phosphate by an independent pathway involving the inducible enzyme glycerol kinase and a flavin-linked glycerol 3-phosphate dehydrogenase.

The drastic curtailment of the anaerobic pathway for glycerol is apparently achieved through the concerted effects of respiratory repression and the inactivation of the dehydrogenase, resulting in a shortage of the probable inducer, dihydroxyacetone (17, 18). Why it is vital for the cell to switch so rapidly and effectively from the dehydrogenase pathway to the glycerol kinase pathway when growth shifts from anaerobic to aerobic conditions can at this stage of our knowledge only be conjectured. One possibility is that in the presence of air, the operation of the de-

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hydrogenase pathway may give rise to harmful amounts of methylglyoxal (4, 33).

The aim of this work was twofold: (i) to purify the dehydrogenase of *K. aerogenes* to homogeneity and to characterize some of its physical properties; and (ii) to prepare specific antibodies against this enzyme in order to track the protein during the inactivation process.

MATERIALS AND METHODS

Chemicals. The sources of organic and inorganic chemicals as well as growth medium supplements are the same as those previously reported (17). DEAE-cellulose (DE-52) was obtained from H. L. Reeve Angel and Co.; Sepharose 6B was from Sigma Chemical Co.; and the Sephadexes were from Pharmacia Fine Chemicals, Inc. Polyacrylamide gel reagents were products of Eastman Organic Chemical Co. Goat anti-rabbit immunoglobin G (IgG) antiserum was purchased from Cappel Laboratories, Inc. Cibacron blue F3GA dye was a gift from Ciba Geigy Corp. Aquasol, L-[*U*-^{14}C]leucine (324 mCi/mmol), and L-[3,4,5-^{3}H(N)]leucine (5 Ci/mmol) were obtained from New England Nuclear Corp.

Bacterial strains and growth conditions. *K. aerogenes* strain 2103 was used to purify the dehydrogenase and for in vivo studies of enzyme inactivation (18). Cultures used for enzyme purification were grown in MS (minimal salts) medium (24) supplemented with 0.03% casein hydrolysate (vitamin-free) and 20 mM glycerol in flasks filled to the top and loosely capped to allow the release of gas. Cultures were incubated at 37°C with slow magnetic stirring until the cells reached the late exponential phase of growth (90 Klett units with a no. 42 filter; 1 Klett unit = 4×10^6 cells/ml).

Enzyme purification. Two liters of a bacterial culture in late exponential phase of growth was harvested by centrifugation in the cold, washed with 250 ml of MS medium (4°C), and suspended for sonication in 5 ml of 0.1 M potassium phosphate at pH 7.0. The cells were disrupted by 8 min of sonication in a 60-W disintegrator (Measuring and Scientific Equipment), during which time the suspension was cooled by an ethanol-Dry Ice bath. The sonic extract was centrifuged at 20,000 × *g* for 15 min (4°C) to remove the debris, and the resulting supernatant was termed "crude extract."

Ammonium sulfate precipitation of the dehydrogenase from crude extract (diluted to a protein concentration of 15 mg/ml) was performed by slow stepwise addition of solid ammonium sulfate until it was brought to 30% saturation. The precipitate was collected by centrifugation. The supernatant fraction was then brought to 55% ammonium sulfate saturation; after slow stirring at 4°C for 60 min, the precipitate was collected by centrifugation and dissolved in 4 ml of 0.05 M potassium phosphate at pH 7.0 (buffer K).

The dissolved ammonium sulfate precipitate was applied to a column of Sepharose 6B (300 ml; 2.6 by 90 cm) equilibrated with buffer K, and the eluate was collected in 1.5-ml fractions. Tubes containing significant dehydrogenase activity were pooled, and the combined fractions were applied to a column of DE-52 equilibrated in buffer K. After washing with 100 ml of equilibrating buffer, the column was eluted with 500 ml of KCl (0 to 1 M gradient), and the eluate was collected in 5-ml fractions. Tubes containing the highest specific activity were pooled and concentrated by pressure dialysis (PM-10 Amicon filter), and the concentrate was dialyzed overnight against 100 volumes of buffer K containing 20 mM glycerol.

Further purification of the dehydrogenase was carried out by affinity chromatography using a matrix of Cibacron blue F3GA, which was covalently bound to Sepharose 6B (blue-Sepharose) by the method of Böhme et al. (3). The dialyzed enzyme preparation was applied to a column of the blue-Sepharose with ample capacity to bind all of the active enzyme. After the column was washed with three times its volume of buffer K containing 20 mM glycerol, the enzyme was eluted with an equal volume of buffer K containing 2 mM NAD^+. The pooled NAD^+ eluates were concentrated, and most of the free pyridine nucleotide was removed by pressure dialysis (PM-10 Amicon filter). The removal of NAD^+ prevents the formation of dihydroxyacetone, which may cause enzyme inactivation during storage.

Protein concentrations were determined in these studies by the biuret method (13).

Determination of molecular weights. Estimates of the molecular weight of the dehydrogenase, with appropriate protein standards, were performed by Sepharose 6B gel chromatography (1) and by electrophoresis (7) in gels containing 5, 6, 7, and 8% acrylamide at pH 8.7 (Tris-glycine). Estimates of subunit molecular weight were made by using 10% acrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) (28). Gels were stained for protein with Coomassie brilliant blue and for the dehydrogenase activity by incubating in 0.5 M sodium carbonate (pH 9.0) containing 10 mM glycerol, 10 mM NAD^+, 50 μg of nitroblue tetrazolium per ml, and 100 μg of *N*-methylphenazonium methosulfate per ml.

Labeling of the enzyme. An anaerobic culture grown overnight was used to inoculate (at a cell density of 10 Klett units) 250 ml of fresh medium containing minimal salts, 20 mM glycerol, 15 mM sodium pyruvate, and 0.01% casein hydrolysate, plus either 500 μCi of [^{3}H]leucine (0.4 μM) or 50 μCi of [^{14}C]-leucine (0.6 μM). Anaerobic conditions were maintained by slowly sparging the cultures with a gas mixture of 95% N_2 + 5% CO_2. When growth reached a density of 80 Klett units, further labeling was stopped by the addition of 60 μg of cold leucine per ml, 10 mM glucose, and 8 μg of chloramphenicol per ml to each of the two cultures. A sample was withdrawn from each culture and harvested (time zero) to determine the specific activity of the dehydrogenase and to precipitate labeled enzyme with specific antiserum. The remainder of the ^{14}C-labeled culture was divided into 150-ml lots and incubated aerobically by vigorous shaking in 2-liter Erlenmeyer flasks for 3 to 4 h. The remainder of the ^{3}H-labeled culture was incubated without division under 95% N_2 and 5% CO_2 for the same period. Crude extracts of labeled cells (twice washed with MS medium at 4°C) were prepared as described above.

Preparation of antibodies. Antibodies against

the dehydrogenase were obtained from rabbits given a series of three subcutaneous injections of 100 μg of the enzyme eluted from the blue-Sepharose column and purified further by electrophoresis in 7% polyacrylamide gels. Location of the enzyme in these gels for recovery by elution was carried out by staining similar gels electrophoresed in parallel for protein and for catalytic activity. Primary immunization consisted of a Freund adjuvant mixture of the enzyme, whereas boosters, given subsequently at 2-week intervals, were composed of an enzyme saline solution. Specific antibody levels were measured every 4 days after the second booster, and large-volume bleeding was performed when titers reached a maximum, usually 8 to 16 days after the second booster. Antibody titers were measured by determining the amount of serum required to inhibit a standard amount of enzyme activity; 1 to 2 μl of high-titer serum was found to inhibit 5 U of enzyme activity. Serum from individual rabbits was used separately.

Antibody precipitation of the labeled enzyme. Specific antiserum was first pretreated with extracts of cells grown aerobically in glucose MS medium. This pretreatment was found to remove antibodies that were reactive with bacterial proteins other than the dehydrogenase. Fully active ^{14}C- or ^{3}H-labeled enzyme (in the zero-time samples before the aerobic incubation experiment) was precipitated from crude extracts by the addition of an amount of pretreated antiserum which was 1.5-fold in excess of that needed to inhibit the total dehydrogenase activity. To precipitate the labeled dehydrogenase protein from extracts containing partially inactivated enzyme, the amount of antiserum added was the same as that used for treating the zero-time sample. All mixtures of extracts and antiserum were incubated for 4 h at 25°C, after which the precipitates were collected by centrifugation and washed twice with an amount of physiological saline (4°C) equivalent to the original volume of the mixture. Second antibody immunoprecipitations, using goat antiserum prepared against rabbit IgG, were performed by adding goat serum at the end of the initial 4-h incubation, and precipitates were allowed to form for an additional 2 h at 25°C. The amount of goat antiserum necessary for maximal precipitation of the rabbit antibodies was predetermined for each incubation mixture. Radioactivity in washed immunoprecipitates dissolved in 0.1 ml of 0.5 M NaOH was measured by counting in 10 ml of Aquasol liquid scintillation cocktail.

Enzyme assays. The dehydrogenase activity was measured spectrophotometrically by following the glycerol-dependent formation of NADH at 340 nm (17). The initial concentration of NAD^+ in the assay mixture was 0.6 mM. Dihydroxyacetone kinase activity was determined by a binding assay in which ^{14}C-labeled dihydroxyacetone phosphate was quantitatively recovered on DEAE-cellulose filters as described previously (17). Units of enzyme activity are expressed in micromoles per minute.

RESULTS

Preparation of the enzyme. Previous studies on the dehydrogenase from *K. aerogenes* described purification procedures that yielded enzyme of 85 to 90% purity (12). Because we wanted to use immunological techniques for analyzing the inactivation of the dehydrogenase, we had to obtain a homogeneous preparation of the enzymatically active protein. By the simplified four-step purification described in Methods and Materials and outlined in Table 1, 12% recovery of electrophoretically homogeneous enzyme was achieved (Fig. 1). The first three steps were similar to those used in previous reports and yielded enzyme of similar purity. Homogeneous enzyme was isolated in the final step by affinity chromatography using a matrix of Cibacron blue F3GA dye bound to Sepharose 6B (blue-Sepharose). Cibacron blue was shown to have a structural resemblance to the adenosine moiety of the cofactors NAD^+ and ATP, and is therefore useful for purifying a variety of dehydrogenases and kinases (25). When the dehydrogenase purified by DE-52 chromatography was applied to a column of blue-Sepharose equilibrated with a buffer containing glycerol, active enzyme was quantitatively eluted with the wash solution containing either NAD^+ ($\geq$1 mM) or ATP ($\geq$4 mM). The enzyme was resistant to elution with 1 M ammonium sulfate or 1 M KCl. The final enzyme preparation was subjected to pressure dialysis both to concentrate the protein and to remove most of the NAD^+ and glycerol.

Estimates of molecular weight. After purification by affinity chromatography, the dehydrogenase recovered was subjected to gel electrophoresis (pH 8.7) as described in Materials and Methods. The relative mobility of the enzymatically active protein in gels of 5 to 8% acrylamide was compared with those of standard proteins. A molecular weight of 79,000 was indicated for the dehydrogenase. Gel filtration through Sepharose 6B in pH 7.0 phosphate buffer, in contrast, revealed a molecular weight of approximately 180,000. A high-molecular-weight form was also observed during polyacrylamide gel electrophoresis at pH 7.2 under nondissociating conditions (results not shown). Electrophoresis in polyacrylamide gels containing SDS, however, showed a single protein band of 40,000 molecular weight. Enzymatically active glycerol dehydrogenase therefore appears to exist as a dimer or tetramer.

In vivo inactivation of the enzyme. Since previous findings indicate that this process requires an exogenous carbon and energy source (9), the rates of in vivo inactivation of the dehydrogenase were examined under well-aerated conditions in the presence of different sources of energy and carbon. The inactivation occurred in the presence of either glucose or glycerol (Fig. 2). In 45 min, half of the total enzyme activity

TABLE 1. *Procedures for the purification of glycerol dehydrogenase from K. aerogenes strain 2103*

Procedure	Total protein (mg)	Total activity (μmol/min)	Sp act (U/mg of protein)	Purification (fold)	Recovery (%)
Crude extract	560	600	1.1		100
Ammonium sulfate (30–55%)	250	860	2.4	2.3	140
Sepharose 6B	29	450	16	15	75
DE-52	3.2	110	35	33	25
Blue-Sepharose	1.5	71	49	46	12

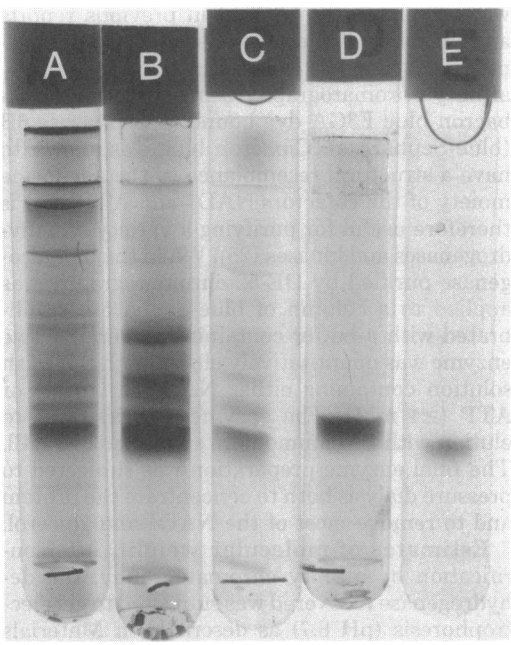

FIG. 1. *Polyacrylamide gel electrophoresis of glycerol dehydrogenase. Samples at various stages of purification (Table 1) were electrophoresed in gels of 7.5% acrylamide. (A) 50 μg of protein from 30 to 55% $(NH_4)_2SO_4$ step; (B) 70 μg of protein from Sepharose 6B step; (C) 50 μg of protein from DE-52 step; (D) 50 μg of protein from Sepharose step; (E) 20 μg of protein from blue-Sepharose step. Gels A through D were stained for protein with Coomassie brilliant blue, and gel E was stained for glycerol dehydrogenase activity as described in Materials and Methods.*

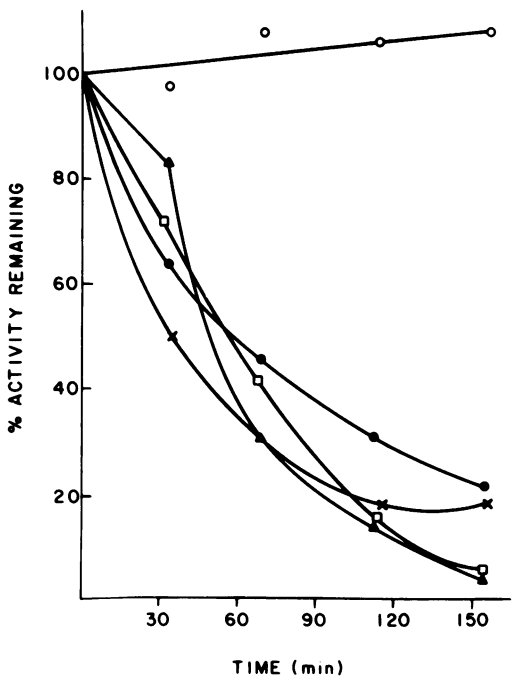

FIG. 2. *Rates of in vivo inactivation of glycerol dehydrogenase. Cells grown anaerobically to late exponential phase in MS medium containing glycerol were harvested, washed, and suspended in fresh MS medium (150 ml in 2-liter flask) to which various test compounds were added. Cell suspensions (at an initial density of 20 Klett units) were vigorously shaken at 37°C, and portions were removed at the times indicated for sonication and measurements of glycerol dehydrogenase activity. Total enzyme activity in aerobically incubated cultures was corrected for dilution of the dehydrogenase activity produced by increases in cell mass per unit volume. Symbols: (○) MS medium; (×) MS medium + 20 mM glycerol; (▲) MS medium + 10 mM glucose; (□) MS medium + 10 mM glucose + 8 μg of chloramphenicol per ml; and (●) MS medium + 10 mM glucose + 1.5 mM phenylmethylsulfonyl fluoride.*

disappeared. Similar inactivation occurred in the presence of succinate or gluconate, provided the cells were preinduced with these compounds for about 3 h (data not shown). Cells which were washed and suspended in MS medium, in contrast, retained full enzyme activity.

The addition of a serine protease inhibitor, phenylmethylsulfonyl fluoride, at a concentration capable of inhibiting protein breakdown (16) had little effect on the rate of enzyme inactivation in cells supplied with glucose. The addition of chloramphenicol (10 μg/ml) also had an insignificant effect on the rate of inactivation, confirming an earlier observation (9). (For unknown reasons, the in vivo rates of inactivation varied slightly from one cell preparation to another.) Under conditions in which 95% of the dehydrogenase was inactivated by aeration, only

5% of dihydroxyacetone kinase activity was lost (Table 2).

Conservation of cross-reacting material during inactivation of the enzyme. To find out whether inactivation of the enzyme involves extensive breakdown of the protein, one culture was grown anaerobically in the presence of [^{14}C]leucine, and another culture was grown in parallel in the presence of [^{3}H]leucine. Samples of the ^{14}C-labeled cells were then incubated aerobically, and samples of the ^{3}H-labeled cells were incubated anaerobically in a medium containing glucose and chloramphenicol. After 240 min at 37°C, the cells were collected and disrupted. The amount of ^{14}C- or ^{3}H-labeled glycerol dehydrogenase protein in the extract was determined by precipitation with specific rabbit antiserum. To ensure complete precipitation of the labeled antigen, goat anti-rabbit IgG was added to another set of samples that contained the dehydrogenase previously exposed to specific rabbit antisera. With both procedures, the recoveries of the ^{14}C- and ^{3}H-labeled glycerol dehydrogenase proteins were similar (about 80% of the radioactivity recovered from zero-time samples; Table 3). In contrast, the dehydrogenase activity remaining in the aerobically incubated cells was only 25% of that in anaerobically incubated cells, which was undiminished. Thus, the loss of enzyme activity upon addition of oxygen could not be accounted for by extensive proteolytic degradation.

TABLE 2. *Effects of in vivo aerobic incubation on the activities of glycerol dehydrogenase and dihydroxyacetone kinase*

Additions to medium of incubation[a]	% Inactivation	
	Dehydrogenase	Kinase
None	0	0
Glucose + chloramphenicol (10 μg/ml)	85	5
Glycerol + chloramphenicol (10 μg/ml)	81	4

[a] Cells grown anaerobically on glycerol were collected, washed, resuspended at a density of 20 Klett units in 150 ml of MS medium, and vigorously shaken for 240 min at 37°C in 2-liter flasks. The cells were then disrupted by sonication, and the specific enzyme activities were assayed.

Electrophoretic properties of the inactivated enzyme. To explore the possibility of a more moderate change in the enzyme associated with its inactivation, immunoprecipitates of inactive ^{14}C- and active ^{3}H-labeled glycerol dehydrogenase were coelectrophoresed in polyacrylamide gels containing SDS to detect possible differences in their subunit molecular weights. No significant difference was found (Fig. 3). Furthermore, the mobility of ^{14}C- and ^{3}H-labeled enzyme proteins recovered from unincubated control extracts was indistinguishable from that of unlabeled enzyme purified to homogeneity (data not shown).

To see whether aerobic inactivation imposed a change in net charge on the subunits of the enzyme, an immunoprecipitate containing a mixture of the inactive ^{14}C-labeled dehydrogenase (80% inactivated) and the active ^{3}H-labeled dehydrogenase was subjected to isoelectric focusing in the presence of 4 M urea (29) to dissociate the enzyme proteins from the antibodies. No significant difference was detected by this approach (Fig. 4). Subunits of both the active and

TABLE 3. *Effects of in vivo aerobic and anaerobic incubation on the recovery of labeled glycerol dehydrogenase by immunoprecipitation*[a]

Cell label	Condition of subsequent incubation	Incubation time (min)	Extract vol (μl)	Radioactivity in immunoprecipitates (10^3 cpm)	% of zero-time value
L-[^{14}C]leucine	Aerobic	0	25	27	
	Aerobic	240	25	22	82
	Aerobic	0	50	51	
	Aerobic	240	50	42	82
	Aerobic	0	25[b]	28	
	Aerobic	240	25	24	86
L-[^{3}H]leucine	Anaerobic	0	25	100	
	Anaerobic	240	25	79	79
	Anaerobic	0	25[b]	110	
	Anaerobic	240	25	88	80

[a] Cells labeled as indicated were grown anaerobically on glycerol, after which portions of the cells were harvested (zero time) and the remainder were incubated aerobically or anaerobically for 240 min. Preparation of extracts and precipitation of labeled enzyme with specific rabbit antibodies were performed as indicated in Methods and Materials.

[b] With added goat anti-rabbit IgG.

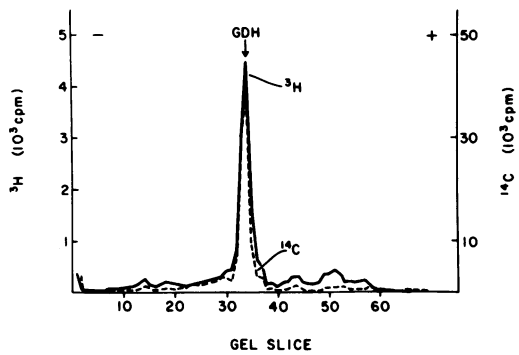

FIG. 3. *SDS-polyacrylamide gel electrophoresis of immunoprecipitated ^{14}C-labeled inactive and ^{3}H-labeled active glycerol dehydrogenase. Extracts were prepared from ^{14}C-labeled cells that had been incubated aerobically and ^{3}H-labeled cells that had been incubated anaerobically for 240 min. After precipitation of labeled glycerol dehydrogenase from a mixture containing equal amounts of ^{14}C- and ^{3}H-labeled proteins, the immunoprecipitates were dissolved in 4 M urea containing 1% SDS and electrophoresed in 10% polyacrylamide gels containing 0.1% SDS at pH 7.2. Gel slices were prepared and counted for double-label determinations of radioactivity.*

the inactive enzyme had isoelectric points of about pH 4.7.

Chromatographic properties of the active and inactivated enzyme. The sizes of active and inactive glycerol dehydrogenase were compared under a nondenaturing condition by chromatography through a column of Sephadex G-200. A mixture of crude extracts containing the inactive ^{14}C-labeled dehydrogenase and the active ^{3}H-labeled dehydrogenase was applied. Eluate fractions containing high glycerol dehydrogenase activities had slightly elevated ^{3}H/^{14}C ratios (Fig. 5), suggesting a loss of the inactivated enzyme. Further support for this conclusion was obtained when samples of these pooled fractions (C to E described in Fig. 5), containing most of the dehydrogenase activity, were treated with specific antiserum. The ^{3}H/^{14}C ratio of the collected immunoprecipitates became even greater (data not presented).

To confirm this interpretation, the remaining fractions C, D, and E were each concentrated by pressure dialysis. The antigen in these concentrated fractions was precipitated with specific antiserum, and the labeled precipitates were collected and then dissociated by boiling in SDS. The enzyme proteins and immunoglobulins thus solubilized were then electrophoresed in polyacrylamide gels containing SDS, and the gels were sliced and counted. A mixture of ^{3}H- and ^{14}C-labeled crude extracts before Sephadex G-200 chromatography was treated similarly and used as a control. Gel slices from the immunoprecipitate of fraction D had an elevated ^{3}H/^{14}C ratio (Fig. 6B) not seen in the control (Fig. 6A). Gel slices of immunoprecipitates from pre- and post-peak fractions (groups C and E) also showed some enrichment for ^{3}H-labeled enzyme (data not presented). Oddly, there were no Sephadex G-200 fractions with a decreased ^{3}H/^{14}C ratio, which would indicate an enrichment for ^{14}C-labeled inactive dehydrogenase. This was the case when the fractions were counted directly or when the counts were carried out with the immunoprecipitates.

Fractionation of labeled enzyme by blue-Sepharose chromatography. Our success in using blue-Sepharose affinity chromatography to purify the dehydrogenase suggested another method for resolving ^{3}H- and ^{14}C-labeled enzymes. A mixture of crude extracts containing inactive ^{14}C-labeled dehydrogenase and active

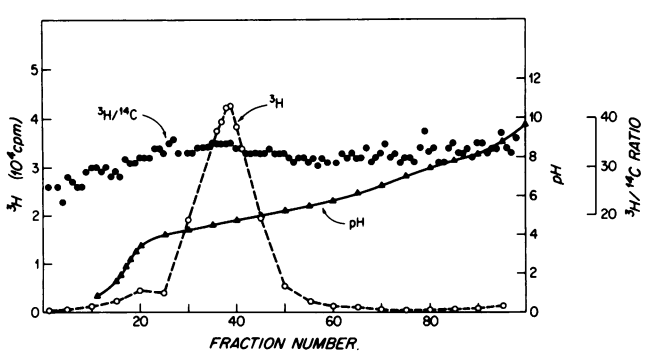

FIG. 4. *Isoelectric focusing in 4 M urea of the immunoprecipitates from mixed extracts containing inactive ^{14}C-labeled and active ^{3}H-labeled enzyme. Extracts and immunoprecipitates were prepared from aerobically and anaerobically incubated cells as described in Fig. 3 and Materials and Methods, and isoelectric focusing was carried out according to Wellner and Hayes (29). After a pH equilibrium was established, fractions were collected and radioactivity was determined. Symbols: (○) ^{3}H counts; (●) ^{3}H/^{14}C ratios; and (▲) pH. The ^{14}C counts showed a similar distribution with a peak which is coincident with the ^{3}H peak (data not plotted).*

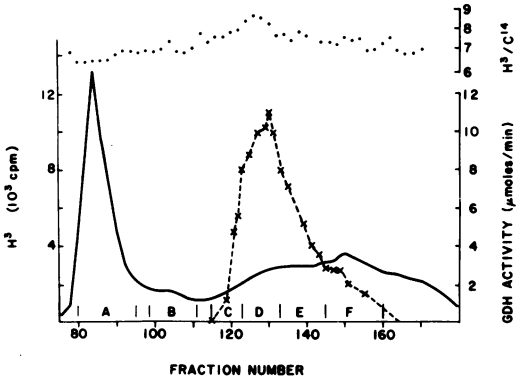

FIG. 5. *Sephadex G-200 chromatography of a mixture of cell extracts containing inactive ^{14}C-labeled and active ^{3}H-labeled enzyme. A mixture of extracts of labeled cells incubated aerobically and anaerobically for 240 min was applied to a column of Sephadex G-200 and eluted with 0.05 M potassium phosphate (pH 7.0) containing 10 mM glycerol. Fractions were counted for radioactivity, and glycerol dehydrogenase activity was measured. Pooled fractions A through F, as indicated by the bars above the abscissa, were then concentrated by pressure dialysis. Symbols: (×) glycerol dehydrogenase activity; (·····) $^{3}H/^{14}C$ ratios; and (——) ^{3}H counts.*

^{3}H-labeled dehydrogenase was applied to a column of blue-Sepharose, and the protein was eluted first with buffer and then with buffer containing 2 mM NAD^+; both eluates were separately concentrated by pressure dialysis. The $^{3}H/^{14}C$ ratio of the NAD^+ eluate containing enyzmatically active dehydrogenase was higher than that of the initial mixture of crude extracts (Table 4). In contrast, the $^{3}H/^{14}C$ ratio of protein not adhering to the column (buffer wash), which represented greater than 90% of the labeled proteins, was significantly lower than that of the starting mixture. More clear-cut results were obtained in a second experiment, in which the $^{3}H/^{14}C$ ratio of the mixture of crude extracts, the buffer wash, and the NAD^+ eluate was determined in the immunoprecipitates. The enrichment for enzymatically active ^{3}H-labeled enzyme in the NAD^+ eluate suggested that active enzyme bound more strongly to this affinity resin. The inactive enzyme appeared not to be bound to the resin, as suggested by the lower $^{3}H/^{14}C$ ratio of the buffer wash and our failure to detect any excess ^{14}C-labeled protein bound to the resin after extensive NAD^+ washing. An attempt to identify the inactive ^{14}C-labeled enzyme by Sephadex G-100 chromatography of concentrated proteins not adhering to blue-Sepharose again failed to reveal a protein fraction enriched for the ^{14}C-labeled species. The failure to recover the inactive dehydrogenase both in this experiment and in the experiment described in the preceding section cannot be explained. It might be due to differential aggregation of this protein, causing its dispersal in different eluate fractions.

Partial restoration of enzyme activity. The belief that aerobic inactivation of the dehydrogenase entails an alteration at the site for the coenzyme was confirmed by the discovery that the protein inactivated in cells supplied aerobically with glucose was restored to about half of its original activity if the assay of the extract was conducted with 10 mM instead of 0.6 mM NAD^+, the standard concentration.

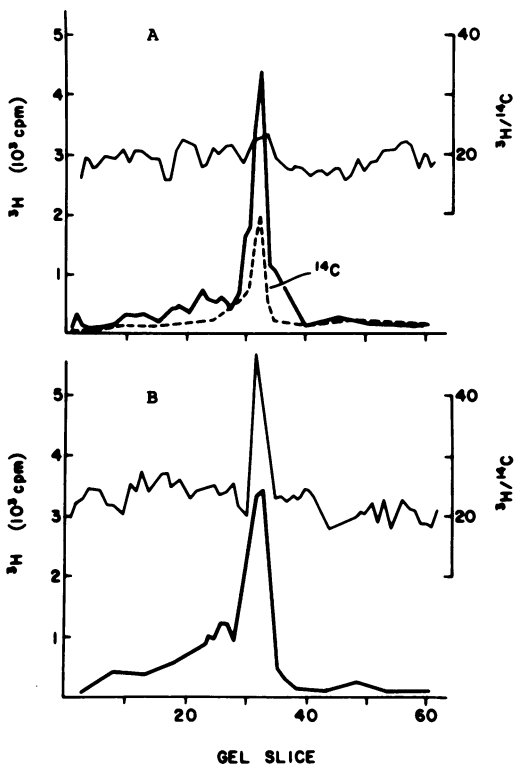

FIG. 6. *SDS-polyacrylamide gel electrophoresis of immunoprecipitates from Sephadex G-200 fractions rich in glycerol dehydrogenase activity. (A) Sample of the mixed cell extract before application to the Sephadex G-200 column (Fig. 5) was treated with rabbit antiserum against glycerol dehydrogenase. The resulting immunoprecipitates were collected and dissolved in 4 M urea containing 1% SDS and electrophoresed in 10% polyacrylamide gels containing 0.1% SDS at pH 7.2. (B) Similar analysis of the concentrated pool D described in Fig. 5. Symbols: (▬) ^{3}H counts; (——) $^{3}H/^{14}C$ ratios; and (- - -) ^{14}C counts.*

TABLE 4. *$^3H/^{14}C$ ratios of eluates from blue-Sepharose columns to which a mixture of inactive ^{14}C-labeled and active ^3H-labeled enzyme was applied*[a]

Fraction	$^3H/^{14}C$ ratio	
	Counted directly	Counted in immunoprecipitate
Mixture of extracts	7.5	4.8
Buffer wash from column	6.5	3.7
NAD^+ eluate	13	19

[a] Results of two separate experiments are shown in which $^3H/^{14}C$ ratios were determined as indicated. In each, crude cell extracts were prepared from cells that had been labeled during anaerobic growth with either L-[^{14}C]- or L-[^3H]leucine and subsequently incubated under aerobic and anaerobic conditions, respectively, for 240 min. A mixture of extracts containing ^{14}C-labeled inactive enzyme and ^3H-labeled active enzyme was applied to a column of blue-Sepharose large enough to bind all of the dehydrogenase protein. The column was eluted first with 0.05 M potassium phosphate (pH 7.0) + 10 mM glycerol (buffer wash) followed by the same solution supplemented with 2 mM NAD^+ (NAD^+ eluate). Eluates which were immunoprecipitated were first concentrated by pressure dialysis.

DISCUSSION

Previous studies of glycerol dehydrogenase from *K. aerogenes* have focused principally on kinetic properties of the enzyme, including its activation by NH_4^+, K^+, and Rb^+, which decrease the apparent K_m of the enzyme for its substrate; definition of an alkaline pH optimum; and inhibition by divalent cations (10, 12, 22). In this work, some of the physical properties of the protein were investigated. The enzyme was shown to be a dimer or tetramer. The subunit has a molecular weight of 40,000 and an isoelectric point of pH 4.7. Under denaturing conditions, it migrates as a single band during electrophoresis. Evidence from Sepharose chromatography and from polyacrylamide gel electrophoresis indicates that the enzyme is capable of forming higher oligomers, but it is not known whether this self-association also occurs in vivo. The extent of purification of the dehydrogenase from crude extracts indicates that the enzyme represents approximately 2% of the soluble protein of *K. aerogenes* cells grown anaerobically on glycerol.

Comparisons of antigenic properties, subunit size, and isoelectric point revealed no marked difference between the active species of the dehydrogenase and the in vivo-inactivated enzyme. Extensive proteolysis, as occurs with yeast malate dehydrogenase, another oxidoreductase, can thus be excluded as the mechanism of enzyme inactivation (2). Specific covalent modification, on the other hand, appears likely. Examination of the enzyme protein indeed showed that the inactive form is altered in its gel filtration (Sephadex G-200). A more striking change in the inactive protein is a decreased affinity for Cibacron blue FG3A dye, "a structural analog" of the NAD^+ cofactor of the enzyme (25). A change in conformation of the enzyme protein at the NAD^+-binding site was revealed more directly by the partial restoration of the lost glycerol dehydrogenase activity when the concentration of the coenzyme in the assay mixture was increased from the standard level of 0.6 mM to 10 mM.

A variety of attempts to reproduce enzyme inactivation in vitro were not fruitful. After a concentrated source of enzyme was mixed with fresh extracts of aerobic cells (50 mg of cell extract protein/ml of incubation medium), no detectable change was observed in the total activity. The addition of nucleotide triphosphates, cAMP, cGMP, NAD^+, NADH, dihydroxyacetone, or one of a number of glycolytic intermediates to crude extracts containing the dehydrogenase also produced no change in activity under aerobic conditions (unpublished observations). Disruption of the cells therefore would appear to dilute critically or destroy a necessary component of the inactivation reaction. Elucidation of the chemical nature of the alteration in the inactive enzyme may require labeling of aerobically incubated cells with radioactive substrates or precursors of potential protein modification reactions, followed by controlled proteolysis and peptide analysis of the purified inactivated protein.

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