SOLUBILIZATION BY CHELATING AGENTS OF A PARTICLE-BOUND INDUCIBLE DEHYDROGENASE*

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Chelating agents such as ethylenediaminetetraacetate (EDTA) are capable of disrupting or functionally altering certain cell structures, e.g., ribosomes (Tissières and Watson, 1958) and the cell walls of bacteria (Repaske, 1956) and plants (Letham, 1960). This communication reports studies in which particles containing an oxidase for D-allohydroxyproline, induced in Pseudomonas striata, have been treated with EDTA to yield a soluble dehydrogenase portion, detected with phenazine methosulfate. The oxidase was earlier found to be associated with a small-particle fraction sedimented at 100,000 x g (Adams, 1959) and more recently (Yoneya and Adams, 1961) the particulate enzyme was shown to be cytochrome linked and stimulated by phenazine methosulfate. The solubilizing action of EDTA suggests a requirement for metal ions in maintaining the structure of such oxidative particles as well as of the other cell components cited above; consistently, it was found (Yoneya and Adams, 1961) that

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the particulate oxidase, assayed without artificial carriers, was stimulated by Ca^{++}, Mg^{++} or Mn^{++} and inhibited by EDTA.

The oxidase appears not to be associated with the ribosome fraction** and, as has been proposed for other particulate bacterial oxidizing systems (for references, see Marr, 1960, and Mudd et al, 1961), may be initially bound to the cell membrane.

Preparation of Soluble Enzyme

Cells of P. striata were grown in mineral media containing 0.2% L-hydroxyproline and 0.05% yeast extract (Difco) as the only organic additions. After harvesting and washing, the cells were broken by sonic lysis (Raytheon 10kc, 10-15 minutes) in 5 volumes of water. The sonic extract was centrifuged for 30 minutes at 25,000 x g and the pellet was discarded. The supernatant solution was centrifuged for 90 minutes at 100,000 x g and the resulting pellet was washed once in water. The washed, drained, ultracentrifugal pellets, stored at -15°, were homogenized in 10 volumes of cold water and shaken for 10 minutes at 30° in 0.05 M glycine, pH 8.8, containing 0.025 M EDTA previously adjusted to pH 9. Treatment with EDTA resulted in solubilization of 60-90% of the enzyme as assayed with phenazine methosulfate.*** The criterion for solubilization was failure

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**EDTA was initially tested for possible solubilizing action, at the suggestion of Dr. Audrey Stevens, with the possibility that the oxidase might be ribosome-associated.

*** Particulate or soluble enzyme was incubated for 10 minutes in a volume of 1 ml containing 0.05 M glycine, pH 9.4, 0.01 M D-allohydroxyproline and 0.8 mM phenazine methosulfate. The reaction was stopped by dilution with cold water and aliquots were assayed for the pyrroline product by measuring the color (550 mμ) developed by heating in an acid solution of p-dimethylaminobenzaldehyde.

to sediment the enzyme on centrifugation at 100,000 x g (40,000 rpm, Spinco Model L Centrifuge) for periods up to 4 hours. Parallel treatment of particles in glycine buffer alone resulted in recovery of virtually all the enzyme as particles sedimentable in 1 hour. Dilute pyrophosphate (0.005 M pH 8.8) also solubilized significant activity. Treatment of the particles with ribonuclease, trypsin, snake venom, urea or detergents failed to release phenazine methosulfate assayable enzyme in soluble form. Mg^{++} (0.05 M), spermine (0.002 M) or spermidine (0.005 M) prevented the solubilizing action of EDTA.

Unlike the particles, which catalyze a slow reaction without added carriers, the soluble enzyme requires phenazine methosulfate; other electron acceptors tested with negative results include methylene blue, ferricyanide, pyocyanine, benzyl and methyl viologen, FMN, FAD, mammalian cytochrome c, and microsomal cytochrome b_5.**** The EDTA-solubilized enzyme has been further purified (about 10-fold) by fractionation with ammonium sulfate and calcium phosphate gel.

Origin of Particulate Enzyme

The enzyme is initially obtained in a fraction containing ribosomes as well as particulate oxidative enzymes. The well-known action of chelating agents both in fragmenting ribosomes and releasing from them several hydrolytic enzymes (for references, see Spahr and Hollingworth, 1961) suggested that the D-allohydroxyproline oxidase system might be initially

****A sample of cytochrome b_5 was kindly provided by Dr. Phillip Strittmatter.

ribosome-bound. Gradient centrifugation (Fig. 1), however, showed definite separation of the peaks for particulate RNA and particulate enzyme; the possibility that enzyme-containing particles are associated with a minor ribosomal fraction, sedimenting separately from the major RNA peak, is not eliminated by these observations.

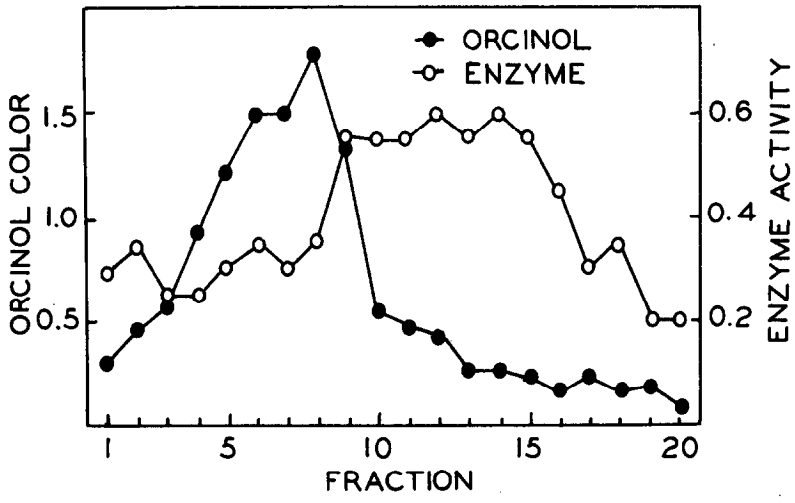

Fig. 1. Distribution of particulate RNA and enzyme after gradient centrifugation. Cells were disrupted in 5 volumes of Tris-succinate-Mg^{++} (TSM) buffer (McQuillen et al, 1959) in a French pressure cell. After discarding material sedimented at 25,000 x g (30 minutes), 0.2 ml of extract was layered (in a linear gradient with 0.2 ml of 4% sucrose) on 4 ml of a 20% to 5% sucrose gradient (Bolton et al, 1959) in TSM buffer. After 90-minute centrifugation at 37,000 rpm in the swinging bucket head of the Model L Spinco, 0.25 ml fractions were collected through a pinhole, fraction 1 being the bottom-most. Aliquots (0.03 ml) were precipitated with 0.2 ml of 7% trichloroacetic acid, washed once with 0.5 ml of trichloroacetic acid, dissolved in NH_4OH and assayed for orcinol color (Mejbaum, 1939). Other aliquots (0.1 ml) were assayed for enzyme as described above. Orcinol color and enzyme activity are expressed as absorbancy at 660 mμ and 550 mμ respectively. We acknowledge with thanks the help of Dr. Gordon Lark in carrying out this experiment.

Relatively gentle methods of cell lysis and extraction (grinding with Alumina A-301 in Mg^{++}-containing buffer) re-

leased considerable enzyme (about 50%) as a large-particle fraction which could be sedimented at 25,000 x g for 20 minutes. Sonic lysis of cells in water, however, yielded enzyme particles which required at least 60 minutes at 100,000 x g for appreciable sedimentation. These observations also are consistent with the conclusion that the oxidase is not ribosomal, but is associated initially with large particles that can be variably comminuted by different extraction procedures.

Several other particulate bacterial oxidases (which were stimulated by phenazine methosulfate and not inhibited by EDTA) have been tested for solubilization by EDTA. Using the specific conditions successful with D-allohydroxyproline oxidase, it was not possible to demonstrate the release of soluble dehydrogenases by EDTA treatment of the succinic oxidases of P. striata, Azotobacter agilis, or Eschericia coli, or of the inducible L-mandelate oxidase of P. fluorescens (Stanier et al, 1953). The present results would suggest the desirability of testing chelating agents more extensively in efforts to solubilize particulate bacterial oxidative enzymes.

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