Physiological and Chemical Properties of a Reductant-activated Inorganic Pyrophosphatase from Desulfovibrio desulfuricans

By D. A. WARE and J. R. POSTGATE
A.R.C. Unit of Nitrogen Fixation, University of Sussex, Brighton, BN1 9QJ, Sussex

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SUMMARY

An inorganic pyrophosphatase was purified from Desulfovibrio desulfuricans strain BERRE SOL. Its activity was increased up to 130-fold by reduction with reagents (Na₂S₂O₅, Na[BH₄], Na₂S, etc.) of $E_\text{h}$ values less than −0.14V at pH 7.0 and 25°C. The pure enzyme was isoelectric at pH 6.55; its molecular weight was 41,600 according to amino acid analysis, consistent with values of 43,000 ± 7000 from ultracentrifugation and exclusion chromatography. The enzyme was very unstable at both 4°C and 15°C and was easily damaged physically: it lost 75% of activity on freezing and thawing and was progressively destroyed if O₂, N₂ or CO were bubbled through its solution. With 6.25 mM-sodium pyrophosphate, the enzyme showed maximum activity at pH 6.2 with 1 mM-CoCl₂ as co-factor; with 3 mM-MgCl₂ or 0.3 mM-MnCl₂ the pH optimum was 8.0. The molecular weight and isoelectric point of the form active without reduction were the same as those of the form active only after reduction; no gross conformational change on reductant activation was detected by spectropolarimetry and fluorescence tests, but reductant activation was associated with an increase in the number of −SH groups in the molecule and a change in electrophoretic mobility at pH 10.

Neither crude nor highly purified enzyme preparations were sensitive to oxygen but the degree of reductant activation of the extracted enzyme depended on the extent to which the intact bacteria had been exposed to oxygen. When bacteria from a continuous culture were harvested with minimum exposure to air they contained active enzyme showing little reductant activation; comparable bacteria resuspended in environments containing dissolved oxygen, but neither a carbon source nor sulphate, yielded enzyme preparations which were almost or completely inactive without a reductant. Addition of sodium lactate and sulphate to the aerated bacteria reversed the phenomenon: the enzyme when extracted was active without reductant. Intracellular inactivation and reactivation processes were rapid and were not influenced by chloramphenicol; alteration of the nutritional status of the population did not affect the intracellular state of the enzyme. Reversible inactivation of inorganic pyrophosphatase was observed in four other strains; it may be a survival mechanism in Desulfovibrio, and in clostridia, which enables such anaerobes to conserve ATP while in aerobic environments.

INTRODUCTION

A number of important metabolic reactions involve the liberation of inorganic pyrophosphate, the hydrolysis of which displaces the equilibrium position in favour of the forward reaction (Jencks, 1962). Examples include biosynthetic processes such as the activation of amino acids or fatty acids and the polymerization of deoxyribonucleotides and ribonucleotides. An example of a reaction of this type in microbial catabolism is the formation of adenosine-5'-phosphosulphate as the initial step in sulphate reduction in Desulfovibrio and Desulfotomaculum species (Peck, 1962).
Inorganic pyrophosphatases are often unstable and sometimes show some degree of reactivation by reducing agents. Gordon (1957) showed that the inorganic pyrophosphatase from rat brain, whose activity was increased 18% by cysteine, was 90% inactivated by dialysis at pH 7.4 and that 42% of this activity was restored by cysteine, but not by NaCN, sodium ascorbate + FeSO₄ or sodium thiosulphate. Comparable inactivation and reactivation occurred when the enzyme was stood for a similar time at pH 4.9 and he attributed inactivation on dialysis to the effect of a small drop in pH at the surface of the dialysis membrane. Seal & Binkley (1957) showed inactivation of inorganic pyrophosphatase from pig brain by a number of metal ions; reactivation occurred with cysteine after treatment with some ions, with ethylenediaminetetraacetic acid (EDTA) with other ions, and in some cases with both. Josse (1966a, b) described a constitutive pyrophosphatase in Escherichia coli but reported no instability; indeed this enzyme was stable at 80°C for 10 min or at room temperature for a year in neutral buffer. Similarly, Kunitz (1952) reported no instability in the inorganic pyrophosphatase from yeast at 5°C although the enzyme denatured above 40°C and was inhibited by Ca²⁺, Co²⁺ or Mn²⁺ when Mg²⁺ was the activating ion.

The workers on yeast and aerobic bacterial pyrophosphatases did not test for reductant activation of their preparations. D'Eustachio, Knight & Hardy (1965), while investigating nitrogen fixation in extracts from Clostridium pasteurianum, reported that pyrophosphatase activity was increased severalfold by reductants. Reductant activation of the enzyme had no specific association with nitrogen fixation, and the property was observed in two other clostridia but not in several aerobic microbes. Active reductants included K[BH₄], cysteine, sodium dithionite and sodium ascorbate. No instability was reported.

An inorganic pyrophosphatase was purified from the HILDENBOROUGH strain of Desulfovibrio desulfuricans (now reclassified as D. vulgaris: Postgate & Campbell, 1966) by Akagi & Campbell (1963) but they reported neither instability nor reductant activation. Baliga, Vartak & Jagannathan (1961) reported two inorganic pyrophosphatases from the same strain of Desulfovibrio. One of the enzymes was activated by Co²⁺, the other by Mg²⁺; they mentioned that activity of the Co²⁺-requiring enzyme was increased by sulphite.

We have observed up to 130-fold activation of an inorganic pyrophosphatase from a strain of Desulfovibrio desulfuricans. This paper reports the purification of the enzyme, its characterization and studies on the environmental conditions which determine the state of activation of the intracellular enzyme. A preliminary proposal for the physiological role of reductant-activated pyrophosphatases has been published (Ware & Postgate, 1970).

**METHODS**

Organisms and culture conditions. A nitrogen-fixing strain of Desulfovibrio desulfuricans, strain BERRE SOL (NCIB 8388), was grown for periods of up to 2 years in a chemostat (nominal volume 0.5 l.) of the type described by Baker (1968). The nitrogen-free medium contained (g./l. distilled water): K₂HPO₄, 0.5; Na₂SO₄, 3.8; CaCl₂·3H₂O, 0.2; MgSO₄·7H₂O, 0.7; trisodium citrate, 4.5; sodium lactate, 6.75; 10 ml. of trace element solution; pH adjusted to 7.5 with NaOH or HCl, autoclaved in 10 l. batches at 121°C for 45 min. The trace element solution contained (mg./l. distilled water): FeSO₄·7H₂O, 82; MnSO₄·4H₂O, 8; NaMoO₄·2H₂O, 30; ZnSO₄·7H₂O, 174; H₃BO₃, 232; CoSO₄·7H₂O, 95.6; CuSO₄·5H₂O, 8.

The dilution rate was 0.055 ± 0.003 h⁻¹ at 30°C ± 0.5. The gas phase was N₂ + 20 to 25% (v/v) CO₂ flowing at about 250 ml./min.; the pH of the culture was controlled at 8.0 ± 0.1 by regulating the proportion of CO₂ in the atmosphere; the culture was stirred magnetically without vortex formation. The population density was 0.139 ± 0.005 mg. dry wt bacteria/ml.
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(0.11 mg. bacterial protein/ml.; \( Y_{\text{lactate}} = 2.32 \) g./mole). Effluent of this culture was used for experiments and also to inoculate 20 l. batch cultures of similar medium with the trace element solution omitted but with the addition of (per litre): NH\(_4\)Cl, 1.5 g.; FeSO\(_4\)·7H\(_2\)O, 3 mg.; and yeast extract, 1.0 g. Such batch cultures were stirred for 2 to 3 days at 30°, with the effluent gas from the chemostat passing over. They were harvested before the pH reached 8.0, using a continuous centrifuge at 18,000 rev./min., flow rate 100 ml./min. Batch cultures of other strains of Desulfovibrio were grown at 30° in a similar medium, with 2.5 % NaCl for the marine strain. Clostridium pasteurianum was grown in Hino & Wilson's (1958) medium supplemented with 3 mg. yeast extract/l.

Harvested bacteria from batch or continuous cultures were washed with 20 mM-N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (tes)-KOH buffer, pH 6.5, to remove H\(_2\)S and phosphate, and dripped into liquid nitrogen as a thick slurry. The frozen material was then stored under liquid nitrogen until required. This procedure also disrupted the organisms (Moses, 1955) and thawing yielded a crude extract. Bacteria could be stored at -20° in buffer without damage to the inorganic pyrophosphatase.

**Enzyme purification.** Organisms were thawed, centrifuged at 38,000g for 20 min. and the supernatant liquid decanted and held at 4°. The residue was resuspended in buffer at 4° and kept in ice during further disruption with a Soniprobe (Dawe Instruments Ltd, London) set at 5A for 1 min./g. wet wt. After treatment, the preparation was centrifuged and the supernatant fluids combined. Saturated (NH\(_4\))\(_2\)SO\(_4\) solution (adjusted to pH 7 with NaOH) was added, while stirring, to 40 % of saturation; after standing for 30 min., the preparation was centrifuged and the precipitate discarded. Further (NH\(_4\))\(_2\)SO\(_4\) solution was added to give 60 % saturation of the supernatant fluid. The precipitate (containing 60 % of the total inorganic pyrophosphatase activity) was collected by centrifugation and redissolved in 5 ml. of 20 mM-tris(hydroxymethylamino)methane('tris')-maleate-NaOH buffer (pH 7.2), desalted by passage through a ‘Sephadex’ G25 column (100 x 3 cm.) and the absence of sulphate checked by adding BaCl\(_2\) in N-HCl to a small sample. The desalted enzyme solution was then loaded on a 12 x 2 cm. Whatman DE 52 anion-exchange column, pH 7.2, eluted with the tris-maleate buffer and 5 ml. fractions collected. The enzyme was slightly retarded on the column and emerged as a peak in three fractions immediately after the material appearing in the void volume. The active fractions were combined, concentrated and passed through a ‘Sephadex’ G200 column equilibrated with 20 mM-tes-KOH buffer, pH 6.5. Fractions (5 ml.) containing the enzyme were collected, concentrated and passed through a ‘Sephadex’ G200 column equilibrated with 20 mM-tes-KOH buffer, pH 6.5. Fractions (5 ml.) containing the enzyme were concentrated to approximately 5 ml. in a ‘Diaflo’ ultrafiltration cell (Amicon N.V., Den Haag, Holland), with a membrane retention limit of 20,000 molecular weight.

The concentrated enzyme was then passed through a 10 x 2 cm column of Whatman CM 52 cation exchange cellulose at pH 6.0 in 20 mM-tris-maleate buffer; no retention of the enzyme occurred but contaminating proteins were removed. The fractions containing enzyme were combined, concentrated and passed through a ‘Sephadex’ G200 column equilibrated with 20 mM-tes-KOH buffer, pH 6.5. Fractions (5 ml.) containing the enzyme were collected, concentrated and tested for purity by disc electrophoresis. Because the enzyme was unstable, these operations were completed as rapidly as possible, usually in 18 to 24 h. Storage procedures are discussed below. The enzyme so obtained was pure (see Results) and was used for all experiments except where stated.

**Analyses.** Inorganic pyrophosphatase was assayed in test tubes containing 0.25 ml. 8 mM-CoCl\(_2\), 0.25 ml. 50 mM-Na\(_2\)P\(_2\)O\(_7\), and 0.5 ml. 0.5 M-tris-maleate buffer, pH 6.0, in a water bath at 30°. Nitrogen was bubbled through the mixture to remove dissolved oxygen for at least 10 min. before adding 1 ml. enzyme solution. Sodium dithionite, if necessary, was then added (see below). Six 0.1 ml. samples were withdrawn at approximately 20 sec. intervals after adding enzyme, each added to 2.9 ml. N-H\(_2\)SO\(_4\) and shaken to stop enzyme
activity and to destroy any dithionite (which otherwise intensified the colour obtained in the phosphate assay). Phosphate liberated was assayed by the method of Taussky & Shoor (1953) and its rate of formation was proportional to enzyme concentration. A unit of enzyme activity was that amount which hydrolysed 1 μmole of substrate/min.

To test for reductant activation, 5 to 15 mg. of highly purified sodium dithionite were added immediately after the enzyme, the mixture gently agitated and then sampled as described above. Larger amounts of dithionite inhibited the purified enzyme. The purified dithionite was stored under nitrogen in gas-tight bottles in a desiccator containing silica gel drying agent.

Sulphate was estimated by precipitation as BaSO₄: 1 ml. 0·1 M BaCl₂ + 0·1 ml. concentrated HCl was added to 1 ml. of sample + 0·1 ml. glycerol and the turbidity of the resultant suspension was compared with standards in an EEL Spectra spectrophotometer (Evans Electroelenium Ltd, Halstead, Essex).

Lactate was estimated with p-hydroxydiphenyl as described by Feigl (1966). Protein was estimated by the technique of Lowry, Rosebrough, Farr & Randall (1951).

Sulphydryl groups were estimated by a technique adapted from Cavallini, Graziani & Dupré (1966) using freshly prepared 28·6 μM-cysteine as the standard. A solution of pure inactive enzyme was concentrated to 0·67 mg. protein/ml. 20 mM-tes-KOH buffer, pH 6·8, in the ultrafiltration cell; the effluent from this cell served as the blank. Portions (3 ml.) of the enzyme solution or blank were mixed with 0·1 ml. 0·1 M-sodium EDTA + 2·4 ml. 0·2 mM-tes-KOH buffer, pH 6·8. To the mixtures was added 0·5 ml. of 10 mM-5,5'-dithiobis-(2-nitrobenzoic acid) neutralized with NaOH to pH 7·0, and, after 30 min. at room temperature, the extinction at 412 nm. was read against water. The blank value was subtracted from the value obtained with the enzyme, and the difference was substituted in the equation given by Cavallini, Graziani & Dupré (1966).

The number of sulphydryl groups in the reductant-activated enzyme was obtained by a similar analysis after inactive enzyme had been activated with 100 mg. sodium dithionite/6 ml. enzyme solution and after unreacted dithionite and oxidation products had been removed by repeated dilution and concentration in the ultrafiltration cell until the u.v. spectrum of the effluent was similar to that of the influent buffer. The final effluent was used to prepare the blank.

The protein for amino acid analysis was hydrolysed with 6 N-HCL (Moore & Stein, 1963) and the amino acid composition determined using a Beckman AutoAnalyser.

ATP, ADP, AMP and APS were separated by ascending thin-layer chromatography at room temperature (15° to 18°) on precoated cellulose F plates (E. Merck, Darmstadt, Germany) in n-butanol+acetone+acetic acid+0·9 N-NH₄OH+water (7:5:5:3:2, by vol.) saturated with solid Na₂EDTA. Spots were located under u.v. radiation (257 nm.).

Dissolved Co, Mg and Mn ions were assayed in the A3000 atomic absorption spectrophotometer (Southern Analytical, Camberley, Surrey).

Physical methods. Discontinuous electrophoresis was conducted as described by Hjertén, Jerstedt & Tiselius (1965) in 34 mM-asparagine-tris buffer (pH 7·9), 20 mM-tris-maleate buffer (pH 5·0) or 25 mM-tris (pH 10·0). The gels were set with 0·07 g. ammonium persulphate/100 ml. Molecular weight determination was by meniscus depletion ultracentrifugation using an analytical ultracentrifuge (model E, Beckman Ltd, Palo Alto, California, U.S.A.) with interference optics (Chervenka, 1969) and by ‘approach to equilibrium’ ultracentrifugation in an ‘Omega Christ’ ultracentrifuge (Fa. Heraeus Christ, Osterode-am-Harz, Germany) with Schlieren optics. Isoelectric points were determined by isoelectric focusing in a sucrose-stabilized tris-maleate pH gradient in a cooled apparatus (D. Ware,
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Spectropolarimetry was done in a Jasco ORD/UV/vis instrument (Japan Spectroscopic Co. Ltd, Tokyo, Japan) with a circular dichroism attachment. Fluorescence spectra were obtained in an Aminco-Bowman spectrophotofluorimeter (American Instrument Co. Inc., Silver Springs, Maryland, U.S.A.). Fluorescence measurements with complexes of anilino-naphthalene sulphonate (Thompson & Yielding, 1968) and protein were made with exciting radiation at 370 nm. The natural dye fluorescence at 515 nm was augmented about 100-fold and shifted to 465 nm when it reacted with protein.

**RESULTS**

Reductant activation of crude extracts

The specific activity of crude extracts (the supernatant fluid obtained after disrupting and centrifuging bacterial suspensions) was usually increased by adding 50 mM-sodium dithionite. The degree of activation differed from preparation to preparation but, with no special precautions in handling the bacteria, was generally three- to sixfold, though up to 30-fold increases were sometimes found; a few preparations showed no reductant activation. Low or zero reductant activation in extracts was associated with high specific activity of the unreduced preparations. The variability in the reductant activation of extracts was later traced to the extent to which the live populations had been exposed to air before extraction.
(Ware & Postgate, 1970); once this point was understood, very inactive preparations could be obtained which could be activated more than 100-fold by reduction (Fig. 1).

For the rest of this paper, inorganic pyrophosphatase activity observable without addition of reductants will be ascribed to 'active' enzyme, and that proportion of activity observable only after addition of reductants will be ascribed to 'inactive' enzyme. The sum of these activities will be termed 'total activity'.

Similar reductant activation occurred in extracts of *Desulfovibrio desulfuricans* strain ESSEX 6 (NCIB 8307) and the marine strain NORWAY 4 (NCIB 8310), *D. vulgaris*, strain HILDENBOROUGH (NCIB 8303), *D. gigas* (NCIB 9332) and *Clostridium pasteurianum* (strain w).

Table 1. Yields at various steps in purification of inorganic pyrophosphatase from *Desulfovibrio desulfuricans*

The enzyme was unstable both in crude extracts and when highly purified, therefore total activity and specific activity declined with the time taken for each purification step.

Values quoted are averages of three preparations (for definition of specific activity see text).

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml.)</th>
<th>Protein (mg./ml.)</th>
<th>Specific activity (units/mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cell-free extract</td>
<td>80</td>
<td>16:7</td>
<td>Before reduction: 6; After reduction: 19; Recovery: 100</td>
</tr>
<tr>
<td>2. (NH₄)₂SO₄ precipitation and desalting</td>
<td>27</td>
<td>13:3</td>
<td>Before reduction: 9:7; After reduction: 39; Recovery: 55</td>
</tr>
<tr>
<td>3. Eluate from DE 52</td>
<td>76</td>
<td>0:84</td>
<td>Before reduction: 79; After reduction: 120; Recovery: 30</td>
</tr>
<tr>
<td>4. Eluate from CM 52</td>
<td>19</td>
<td>1:50</td>
<td>Before reduction: 105; After reduction: 240; Recovery: 27</td>
</tr>
<tr>
<td>5. Eluate from G200 and overnight storage</td>
<td>27</td>
<td>0:37</td>
<td>Before reduction: 197; After reduction: 320; Recovery: 12:5</td>
</tr>
</tbody>
</table>

Characterization of the pure protein

**Purity.** The purification technique described above increased specific activity 17-fold over the crude extract (Table 1). Disc electrophoresis of the purified enzyme, after reduction with dithionite, gave a single band migrating in 6% acrylamide gels toward the anode at pH 7-9 and 10-0, and towards the cathode at pH 5-0. At pH 7-9 it gave a single band in 9% acrylamide gels. Preparations which had not been reduced with dithionite and which included both active and inactive enzyme, gave single bands in all these conditions except at pH 10-0, when distinct splitting into two bands less than 1 mm. apart was observed. The band, when cut from a gel at pH 7-9 and eluted with 20 mM-tes-KOH buffer, pH 6-5, retained pyrophosphatase active and inactive components in their original ratio, provided that all the persulphate and tetramethylethylenediamine had been removed by prior electrophoresis of the gel. Enzyme from gels run at pH 5 or pH 10 was irreversibly inactive, as would be expected from its instability at these pH values (see below). A solution of 10 mg. purified enzyme/ml. 20 mM-tes-KOH buffer, pH 6-8, sedimented as a single, symmetrical Schlieren peak at 200,000g and 15°. As would be expected from the purification procedures used, the enzyme, whether partly or wholly active, gave a single peak on elution from (i) a CM 52 cation exchange column equilibrated and eluted with 20 mM-tris-maleate buffer, pH 6-0, (ii) a DE 52 anion exchange column equilibrated and eluted with 20 mM-tris-maleate buffer, pH 7-2, and (iii) a Sephadex G 200 column eluted with 20 mM-tes-KOH buffer, pH 6-5. During a given purification the ratio of inactive to active enzyme remained constant within experimental error (Table 1). These criteria indicated that the pyrophosphatase was a single pure protein.
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which existed in two forms, active and inactive, which had distinguishable electrophoretic mobilities at pH 10.

Molecular weight. Exclusion chromatography on Sephadex G 200 indicated a molecular weight between 35,000 and 45,000. A single determination by meniscus depletion equilibrium ultracentrifugation gave a value of 38,000 ± 5000. This figure is in reasonable agreement with the value of about 41,600 from amino acid analysis (see below) and values of 40,000 to 50,000 from 'approach to equilibrium' ultracentrifugation.

Isoelectric point. The ion exchange properties indicated a slightly acid value; gradient electrofocusing gave a value of pH 6·55.

Michaelis constant. Reaction rate determinations with protein of specific activity 162 units/mg. at 600 μg./tube using pyrophosphate molarities differing by twofold steps between 0·6 and 6·25 mM gave, after the conventional double reciprocal plot, a $K_m$ value of 1·2 ± 0·2 mM $P_2O_5^{4-}$ at pH 6·0.

Catalytic centre activity. Freshly prepared pure enzyme (specific activity 391 units/mg.) hydrolysed $1·5 \times 10^4$ moles pyrophosphate/min./mole when reduced with dithionite.

Table 2. Activation of Desulfovibrio inorganic pyrophosphatase by reductants

<table>
<thead>
<tr>
<th>Reductant</th>
<th>Enzyme activity (units/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0·62 to 0·67</td>
</tr>
<tr>
<td>Na ascorbate</td>
<td>0·78</td>
</tr>
<tr>
<td>Na thioglycollate</td>
<td>0·93</td>
</tr>
<tr>
<td>$Na_2S_2O_3$</td>
<td>1·71</td>
</tr>
<tr>
<td>Cysteine</td>
<td>1·86</td>
</tr>
<tr>
<td>$Na_S$</td>
<td>3·72</td>
</tr>
<tr>
<td>$Na[BH_4]$</td>
<td>5·33</td>
</tr>
<tr>
<td>$Na_2SO_3$</td>
<td>6·82</td>
</tr>
<tr>
<td>$Na_2S_2O_4$</td>
<td>9·30</td>
</tr>
</tbody>
</table>

Activation by reductants. A preparation which contained both active and inactive components (initially pure but having lost some activity during storage: specific activity 190 units/mg.) had its total activity increased 2·6-fold to 14·3-fold by $Na_2S$, sodium dithionite, $Na[BH_4]$, $Na_2SO_3$, $Na_2S_2O_4$ or cysteine, but not by sodium ascorbate or sodium thioglycollate (Table 2). These findings indicate that a sulphur-containing reductant was not essential for activation, and imply that a reductant of $E_m^\circ$ less than $-0·15$ V at pH 7·0 and 25° was necessary.

Amino acid composition. Two samples of 41 mg. pure pyrophosphatase were hydrolysed and analysed (Table 3). Tryptophan was determined separately on a similar sample of pyrophosphatase using the method of Opieńska-Blauth, Charezinska & Berbéc (1963); cysteine was obtained from the SH estimation reported below. The molecular weights calculated from the two sets of data were 41,668 and 41,548 respectively.

Attempts to reverse reductant activation. The ratio of active to inactive enzyme differed from batch to batch of bacteria. Enzyme which had been extracted in a partly inactive state was tested with various oxidants in attempts to induce reversible deactivation. Test tubes containing enzyme in 3 ml. of 20 mm-tris-citrate buffer, pH 7·0, plus oxidant, were gently agitated at room temperature to dissolve solid oxidants. At intervals, samples were tested for pyrophosphatase activity in the absence of reductant and with 10, 15, 20 or 30 mg. sodium dithionite per test. Hydrogen peroxide (0·9, 2·4 or 4·5 mM) had no effect over 5 min. Pure $O_2$
bubbled through an enzyme solution halved activity in 30 min.; K₃Fe(CN)₆, KBrO₃, and K₂S₂O₈ (5 and 10 mg./ml.) or 0.05 ml. HIO₄ halved activity in 5 min. but in no case was the inactivation reversed by sodium dithionite. The lowering of activity by O₂ is discussed under 'stability' below.

Fig. 2. Effect of pH on saturation solubilities of CoCl₂ (●), MnCl₂ (○) and MgCl₂ (●) in the presence of 6.25 mM-Na₂P₂O₇ and 125 mM-tris-maleate buffer at 16°.

Table 3. Amino acid composition of inorganic pyrophosphatase from Desulfovibrio desulfuricans strain BERRE SOL

Data from two separate hydrolyses have been corrected for acid decomposition in 20 h. (Crestfield, Moore & Stein, 1963). Corrections applied were 5% for threonine and tyrosine and 10% for serine. For tryptophan and cysteine see text. Results were rounded upwards and given in terms of residues/mol. wt 42,000.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>Methionine</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>37</td>
</tr>
<tr>
<td>Threonine</td>
<td>26 to 28</td>
</tr>
<tr>
<td>Serine</td>
<td>15 to 17</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>24 to 25</td>
</tr>
<tr>
<td>Proline</td>
<td>14 to 15</td>
</tr>
<tr>
<td>Glycine</td>
<td>29 to 30</td>
</tr>
<tr>
<td>Alanine</td>
<td>29 to 31</td>
</tr>
<tr>
<td>Valine</td>
<td>28 to 30</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>9 to 10</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>13 to 15</td>
</tr>
<tr>
<td>Leucine</td>
<td>24</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>6</td>
</tr>
<tr>
<td>Histidine</td>
<td>6 to 7</td>
</tr>
<tr>
<td>Arginine</td>
<td>7</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>13</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>9</td>
</tr>
<tr>
<td>Cysteine</td>
<td>9 to 10</td>
</tr>
</tbody>
</table>

Metal requirements. The two inorganic pyrophosphatase enzymes which Baliga et al. (1961) described in Desulfovibrio vulgaris were a soluble enzyme which showed maximum activity at pH 8.5 and required Mg²⁺, and an insoluble enzyme which showed maximum activity at pH 7.0 and required Co²⁺, Mn²⁺ or Zn²⁺, although the last two ions gave less activity. Akagi & Campbell (1963) reported only a single inorganic pyrophosphatase, in a substrain of the same organism, which required Mg²⁺ or Mn²⁺ for optimum activity at pH 8.0; Co²⁺ gave 65% of the activity obtained with Mg²⁺. The enzyme from Escherichia coli requires Co²⁺, Mg²⁺ or Mn²⁺ but the activity with each metal ion is influenced by pH, molar ratio of metal to pyrophosphate and solubility of the metal pyrophosphate (Josse, 1966a, b). Josse's work shows that a knowledge of the saturation solubi-
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The relative efficiencies of the metal ions as co-factors were compared by choosing metal chloride concentrations and pH values at which all metal ions were equally soluble in the test solution. Table 4 shows that, at pH 6·2, Co²⁺ was most efficient, whereas at pH 7·7 Mg²⁺ gave slightly higher activity than either Co²⁺ or Mn²⁺.

The effect of pH on enzyme activity with the three co-factors was studied at metal chloride concentrations at which no metal pyrophosphates were precipitated (Fig. 3). For the measurement of total activity the enzyme was reduced with dithionite, and the dithionite removed by dialysis for 3 h. against 25 mM-tris-maleate buffer, pH 7·0. The optimum values of metal ion concentration and pH were 1·0 mM at pH 6·2 for Co²⁺, 3 mM at pH 8 for Mg²⁺ and 0·3 mM at pH 7·7 for Mn²⁺, all with 6·25 mM-Na₂P₂O₇. In their optimal conditions, Co²⁺ and Mg²⁺ were about equivalent and Mn²⁺ about 67% as active.

Stability. The enzyme, both when purified and in crude extracts, was very sensitive to physical damage. Bubbling O₂, N₂ or CO through a crude extract (specific activity 12 units/mg.) containing 1·5 mg. protein/ml. 500 mM-tris-HCl buffer, pH 7·0, destroyed 84% of enzyme activity in 30 min. After freezing and thawing, partially purified enzyme solutions (0·1 mg. protein/ml.; specific activity 60 units/mg.) in 5 mM-tris-maleate buffer, pH 6·6, lost 40 to 100% of their activity. Enzyme inactivated by freezing and thawing had a molecular weight of less than 8000 according to molecular sieve chromatography on Sephadex G 200 equilibrated with the same buffer.

Dialysis against distilled water also led to inactivation: 5 ml. of partially purified enzyme solution (5 mg. protein/ml. of 50 mM-tris-HCl buffer, pH 8·0) lost 75% activity when dialysed at 4° against 5 l. for 18 h. Addition of freeze-dried dialysate to the inactivated enzyme solution did not restore activity. No protein was detected in the freeze-dried dialysate nor

<table>
<thead>
<tr>
<th>Metal salt added</th>
<th>pH 6·2 0·05 mM metal</th>
<th>pH 7·7 0·20 mM metal</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoCl₂</td>
<td>1·5</td>
<td>3·3</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0·3</td>
<td>4·0</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>0·5</td>
<td>3·5</td>
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</table>
was any lost from the dialysed sample, therefore the enzyme had been damaged within the
dialysis membrane. Enzymes dialysed against 25 mM-tris-maleate buffer, pH 7.2, for 14 h. at
4° lost only 28 % activity, no more than a non-dialysed control. Gordon (1957) reported
losses of activity of inorganic pyrophosphatase from rat brain on dialysis but obtained partial
reactivation by reductants. Reductants did not reactivate dialysed *Desulfovibrio desulfuricans*
inorganic pyrophosphatase.

Both crude and purified enzyme preparations lost 30 to 70 % of activity when stored in
25 mM-tes-KOH (pH 7.2) or 20 mM-tris-maleate (pH 6.5) buffer for 24 h. at 4° or 15°, with or
without 1 drop of toluene as a bacteriocidal agent. Purified enzyme lost 50 % activity when
glycerol was added to 20 % (v/v) and the solution frozen in liquid N₂ and thawed, but since
no further loss of activity occurred during storage in liquid N₂ for up to 3 weeks, the pro-
cedure was used to conserve enzyme for longer than 24 h.

The effect of pH on stability is shown in Fig. 4. Although total activity declined at all
pH values, some inactive component became active while standing. This instability made for
difficulty in handling and storing the enzyme and is the reason why specific activities of
initially pure preparations varied. The preparation with the highest specific activity obtained
was that used for calculation of the catalytic centre activity (p. 151).

*Molecular nature of reductant activation.* Baliga *et al.* (1961) reported two pyrophos-
phatasas in *Desulfovibrio vulgaris*. It was possible that those preparations obtained in our
work which contained both active and inactive components represented two enzymes, one
wholly reductant-activatable, the other unaffected by reductants. However, the purification
procedure was a result of numerous pilot attempts using ion exchange chromatography,
molecular sieve chromatography, disc gel electrophoresis, isoelectric focusing, salt pre-
Reductant-activated pyrophosphatase

Precipitation, and high speed centrifugation. Often the starting material was a mixture of active and inactive enzyme; in no experiment was any separation into two components, one active and one reductant-activatable, observed. No changes in the ratio of active to total activity occurred on dilution of an enzyme solution with 20 mM-tes-KOH buffer, pH 6.5, from 19 mg. protein/ml to 0.09 mg. protein/ml., though changes would be expected if reductant activation involved reassembly of subunits. Anaerobic or aerobic handling of extracts during preparation and assay made no difference, nor did disruption of a given batch of bacteria by ultrasound, dripping into liquid nitrogen or extrusion from a French pressure cell. Electrophoresis, as described earlier, gave single bands except with partly active enzyme at pH 10; the band from gels at pH 7-9, when cut out and eluted, was active with Co²⁺, Mg²⁺ or Mn²⁺.

Reductant activation is therefore probably the property of a single protein, presumably involving changes in the oxidation state of some components of the molecule, a change reflected in an altered electrophoretic mobility at pH 10.

Fig. 4. The effect of pH on the stability of purified inorganic pyrophosphatase from *Desulfovibrio desulfuricans* kept at 4° for 18 h. pH readjusted to 6.0 with 10% tris-hydroxide or 10% maleic acid before assay. ○, Active enzyme; ●, inactive enzyme; ○, total enzyme.

To test whether activation was associated with gross conformational changes, the circular dichroism in the range 210 to 300 nm. of 0.2 mg. enzyme/ml. 25 mM-tris-HCl buffer at pH 7.55 was measured before and after activation with Na[BH₄]. It was unchanged, with a peak of 0.2 extinction units at 218 nm.; molecular ellipticity \( \langle \theta = 3300(E_1 - E_3) \rangle = 6.04 \times 10^5. \) Since 8-anilino-napthalene sulfonate binds to proteins and exhibits a strong fluorescence influenced by the conformation of the protein (Thompson & Yielding, 1968), this effect was studied with our enzyme. Sodium dithionite lowered the intensity of the fluorescence with enzyme as well as with bovine serum albumin but Na[BH₄] did not interfere. A complex of 0.2 ml. of 0.18 mM dye in distilled water + 2 ml. inactive enzyme at 0.6 mg./ml. (15 μM) in 20 mM-tes-KOH, pH 6.5, showed no change in wavelength or intensity when activated with 3 mg. Na[BH₄]. A second sample of inactive enzyme was activated with sodium dithionite, desalted by passage through a column of Sephadex G25, concentrated to 0.6 mg. protein/ml. by ultrafiltration and treatment with Lyphogel, and finally the absence of dithionite was
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checked by comparison of its u.v. absorption spectrum with that of the original inactive enzyme. The fluorescence spectra with dye were the same.

Sulphydryl groups were estimated in portions of pure inactive enzyme before and after reduction with sodium dithionite. The dithionite and its oxidation products were removed as described in Methods. The inactive enzyme contained 2.81 sulphydryl groups/molecule which increased to 9.66 after reduction. These values make it most likely that the inactive enzyme contained 3 and the active enzyme 9 sulphydryl groups/molecule.

Table 5. Medium used to resuspend live Desulfovibrio desulfuricans, and its effect on the reductant activation of the inorganic pyrophosphatase released by disruption

Except in the first test, bacteria were centrifuged at 38,000 g for 10 min., resuspended and centrifuged twice in the media listed below, then the resuspended organisms were ruptured by dripping into liquid N₂, thawed and assayed for reductant activation as described in Methods. Values quoted are means of five separate experiments. Specific activities of the various preparations are not quoted because other experiments showed that the extent of disruption of the populations was influenced by the pretreatment.

Effect of culture conditions on the state of the freshly extracted enzyme. The ratio of active to inactive inorganic pyrophosphatase in fresh extracts of Desulfovibrio desulfuricans differed from batch to batch of organism, but was similar in samples taken from the continuous culture in a steady state and handled by a standardized procedure. To test for an effect of nutritional status of the organisms on the state of activity of fresh extracts, the composition of the medium entering the chemostat culture was altered to give (1) conditions of sulphate limitation (lactate, 57 mM; sulphate, 22 mM); (2) conditions of lactate limitation (lactate, 45 mM; sulphate, 27 mM); (3) conditions of phosphate limitation (KH₂PO₄, 24 μM) and (4) the 'normal' condition in which both lactate (54 mM) and sulphate (27 mM) were exhausted. Samples (500 ml.) were withdrawn from the growth vessel at each steady state and crude extracts of the bacteria assayed for ratio of active to inactive enzyme. Each 'abnormal' steady state was obtained on three separate occasions, and the ratio of active to inactive enzyme varied from 1:2 to 1:5 randomly over the nine steady states examined. Therefore nutritional status did not affect reductant activation in our test condition. The age of the bacterial population had some effect on the state of the enzyme: samples were withdrawn from the 'normal' continuous culture, and fresh extracts were assayed for pyrophosphatase activity after washing the organisms once in 10 mM tes-KOH buffer, pH 6.5, both immediately after withdrawal from growing culture, and after being held stagnant for 24 h. at 30° anaerobically in sealed vessels. Activation by dithionite fell from 4.5-fold to 1.7-fold while total activity declined by 28%.

Effect of environment of the live bacteria on reductant activation. Bacteria from the continuous culture passed directly into liquid N₂, thawed and assayed contained mostly active
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enzyme. Similar samples of organisms from the continuous culture, resuspended twice in one of the media listed in Table 5, dripped into liquid nitrogen, thawed and assayed, contained variable proportions of active and inactive enzyme. Though neither disrupted bacteria nor purified enzyme were affected by air, exposure of the living organisms to air during harvesting and washing in buffer increased the proportion of inactive enzyme in extracts. Harvesting in the presence of a reductant, or in growth medium, diminished this effect.

It seemed likely that air induced inactivation of the endocellular enzyme and that lactate and sulphate metabolism might reactivate it. A sample from the continuous culture was therefore centrifuged to remove bacteria, and air and CO₂ bubbled through the supernatant until all H₂S had been removed. Bacteria which had been resuspended in this aerated supernatant contained mostly inactive enzyme. Fresh medium (1 ml.) was added to 9 ml. of these bacteria, and after 2 to 3 min. they were disrupted in liquid nitrogen. They contained mostly active enzyme. This reactivation was not affected by 2 μg. chloramphenicol/ml. If the added medium was different from that in which the bacteria had been grown (e.g. medium B of Postgate, 1966) no reactivation occurred and total activity declined.

Reactivation on addition of growth medium may have been the result of reduction of the enzyme by the H₂S produced in the medium by sulphate reduction. This possibility is unlikely because bacteria which had been resuspended in 20 mM-tes-KOH buffer to induce inactivation and then resuspended in a supernatant from the continuous culture which was saturated with H₂S, still contained mostly inactive enzyme. These findings led to the conclusion that exposure to air induced inactivation and metabolism of sulphate and lactate was necessary to reactivate the enzyme.

DISCUSSION

Nature of reductant activation. It is theoretically possible that two inorganic pyrophosphatases existed in our preparations, only one of which was reductant-activated. Baliga et al. (1961) described two pyrophosphatases in Desulfovibrio (desulfuricans) vulgaris strain HILDENBOROUGH, one of which showed signs of reductant activation in that activity was enhanced by sulphite. Their soluble enzyme showed maximum activity at pH 8.5 with Mg²⁺ ions, while the insoluble enzyme showed a pH optimum of 7.0 with Co²⁺. These results are consistent with those shown in Fig. 3 for the enzyme from D. desulfuricans strain BERRE SOL, but we obtained no sign of separation into two enzymes in numerous tests on our preparations; in experiments not reported in detail we found that washed debris from sonically disrupted bacteria contained only slight activity which was similar in all respects to that of the soluble enzyme. Hence the evidence of Baliga et al. for two enzymes in their strain does not apply to our preparations and we conclude that we have been dealing with a single enzyme.

Reducants may reverse an inhibition due to a metal ion, as in the pyrophosphatase from swine brain which is reactivated by cysteine and EDTA (Seal & Binkley, 1957). Our failure to activate the inorganic pyrophosphatase from Desulfovibrio desulfuricans during ion exchange, or molecular sieve chromatography, or dialysis or electrophoresis, eliminates any explanation of reductant activation along these lines.

Reducant activation could be artificial in the sense of Gordon (1957), who attributed the effect of cysteine on the pyrophosphatase from rat brain to the reversal of damage caused by small pH changes which occurred at the membrane surface during dialysis. But in our preparations, Desulfovibrio pyrophosphatase did not acquire increased reductant activation after dialysis. It is difficult to eliminate completely the possibility that reductant activation was an operational artefact but this is unlikely, since the extent of activation could be re-
producibly varied by exposure of whole bacteria to air, while crude and purified extracts were not air-sensitive.

We therefore conclude that reductant activation is an intrinsic property of the inorganic pyrophosphatase of our organism and not an artefact of extraction or handling. Our spectropolarimetry and fluorescence spectroscopy gave no evidence for gross conformational changes in the Desulfovibrio pyrophosphatase, but such tests would not detect a small conformational change such as might occur if a component of the prosthetic site of the enzyme became reduced. The most probable explanation for reductant activation is that a small conformational change occurs which exposes the active site to the substrate. Reduction and breakage of disulphide bonds was probably involved because activation increased the number of exposed \(-\text{SH}\) groups from \(3\) to \(9\)/molecule; such an increase in titratable \(-\text{SH}\) groups should lead to increased mobility of the activated enzyme at alkaline pH values. Though the enzyme decomposed too rapidly at pH \(10\) for the state of the components to be demonstrated, electrophoresis of our preparations at this pH value showed two components in the partially active material whereas there was one after reductant activation. Bühner \\& Sund (1969) implicated \(-\text{SH}\) group recovery in the reactivation of stored yeast alcohol dehydrogenase.

**Metal requirement.** Metal co-factors are not directly involved in the reduction step of activation because, although \(\text{Co}^{2+}\) and \(\text{Mn}^{2+}\) have various oxidation states, \(\text{Mg}^{2+}\) does not. The complexity of the inorganic pyrophosphatase system was illustrated by the exhaustive work of Josse (1966a, b) on the enzyme from *Escherichia coli*, who showed that activity was influenced by the ionic species predominating (e.g. \(\text{P}_2\text{O}_5^{4-}\), \(\text{HP}_2\text{O}_6^{7-}\), \(\text{MgHP}_2\text{O}_7^{-}\), etc.), the solubility of the metal pyrophosphates (both of which parameters are pH-dependent) and the possibility of substrate inhibition. Our aims in this work have been restricted to the questions (i) whether the active and inactive forms show different behaviour towards metal co-factors and (ii) whether these co-factors show specific differences in activity. The experimental conditions chosen were somewhat arbitrary but they answer these points satisfactorily. Fig. 2 and 3 show that the pH optima with various metals were influenced by the solubilities of the metal pyrophosphates but were similar for both active and inactive enzyme. This finding is consistent with our view that only one enzyme is involved. In conditions in which the metal ions were at similar concentrations (Table 4), Mg, Mn and Co ions were about equally effective as co-factors at a neutral pH but Mg and Mn were only about a third as effective as Co at pH 6. Hence specific differences between the metal ions were small and the wide differences obtained in practice – Mn seeming very much less active than Mg or Co – may be attributed to the uniformly low solubility of manganese pyrophosphates and the difficulty of getting adequate Mn into solution.

**Physiological function of reductant activation.** D'Eustachio et al. (1965) remarked that the reductant activation of inorganic pyrophosphatases should ‘be considered in the study of reactions which eliminate pyrophosphate from ATP, such as amino acid activations’. We propose that the property of reductant activation is the expression of a control mechanism in Desulfovibrio, and by implication in other obligate anaerobes, which enables them to survive short periods of exposure to oxygen. In Desulfovibrio, \(\text{SO}_4^{2-}\) is ‘activated’ by ATP by conversion to adenosine-5-phosphosulphate (APS) by ATP-sulphurylase before reduction. Since the products of this reaction are APS and pyrophosphate, then a control mechanism exerted on the equilibrium position by limiting the pyrophosphatase reaction could prevent depletion of the ATP pool by formation of APS during conditions when the conservation of ATP is critical. If this view is correct, extracts of organisms containing ATP-sulphurylase, APS-reductase and inactive pyrophosphatase ought to show reductant-activated AMP formation. Our attempts to show this in crude preparations of inactive organisms were
only partially successful: they were mentioned earlier by Ware & Postgate (1970) but have not been given in detail here because endogenous activation of the enzyme (as APS was reduced to yield sulphide) and ‘scrambling’ of the adenosine phosphates produced make it difficult to obtain reproducible effects in such preparations.

Conditions in which conservation of ATP is of critical importance may occur in an aerobic environment. Grossman & Postgate (1955) showed that Desulfovibrio exposed to an oxidizing environment is still capable of metabolizing pyruvate or lactate (though not of reducing \( \text{SO}_4^{2-} \) to \( \text{H}_2\text{S} \)), and of reducing oxygen via the autoxidizable cytochrome \( \text{c}_2 \), thus maintaining a reducing micro-environment by depletion of the dissolved oxygen. Our organisms did not inactivate the pyrophosphatase when washed with growth medium, or with aerated medium containing 10% of fresh growth medium (which contained dissolved oxygen and no reductants), presumably because they could still sustain a reducing micro-environment. When washed with medium from which carbon source and sulphate had been omitted, the enzyme was inactivated, but was reactivated within minutes when these substrates were supplied. These experiments provide strong evidence that the state of the pyrophosphatase is under immediate and rapid control in the living organisms.

It seems logical, therefore, to accept that it is of some advantage to Desulfovibrio to conserve ATP in an aerobic environment, and that ability to regulate its pyrophosphatase activity rapidly is a physiological mechanism for doing this. An impressive feature is the rapidity with which responses occur in the living bacteria, which implies a fineness of control rare in prokaryotes. The question why ATP conservation should be critical in an aerobic environment invokes the major problem of why Desulfovibrio is so exacting an anaerobe, and our findings do not advance understanding of this question. But our observations provide a rationalization of reductant activation of inorganic pyrophosphatase in terms of their anaerobic habit. Since reductant activation of inorganic pyrophosphatase was described first among other anaerobes (D'Eustachio et al. 1965) we have proposed elsewhere (Ware & Postgate, 1970) that it may represent a distinctive control process of general importance in other anaerobes also.

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REFERENCES


