L-(+)-Tartrate dehydratase from *Pseudomonas putida* is an iron-sulphur enzyme

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The enzyme L-(+)-tartrate dehydratase has been isolated from extracts of *Pseudomonas putida* by a one-step procedure involving dye-ligand chromatography. The enzyme loses activity rapidly in the absence of Fe^{2+}; concentrated solutions have a brown colour typical of iron-sulphur proteins. Analysis of iron and acid-labile sulphide indicated 3–5 atoms of each per molecule of 100 kDa. The enzyme's structure consists of four subunits, two each of 23 and 27 kDa.

Tartrate dehydratase *Iron-sulfur protein* (Pseudomonas) *Dye-ligand chromatography* *Dehydratase*

1. INTRODUCTION

The role of Fe^{2+} in the activation of aconitase has recently been clarified [1,2]. The molecule has an unstable Fe_4S_4 cluster which, on losing one iron atom, causes loss of activity of the enzyme, and this is reversible. Several other dehydratases, particularly those working on the α, β carbons of a hydroxycarboxylic acid, have been reported to be activated by iron, and it has been suggested that at least some of these may be iron-sulphur enzymes like aconitase [3,4]. We have reported that 6-phosphogluconate dehydratase isolated from *Zymomonas mobilis* has at least 3 iron atoms per subunit and has the characteristic brown colour of iron-sulphur proteins [5]. Maleate hydratase is another enzyme in this category [4].

Of the tartrate dehydratases described in the literature, the L-(+)-tartrate enzyme from *Pseudomonas putida* was demonstrated to be activatable by Fe^{2+} plus large concentrations of sulphydryl compounds [6]. The D-(−)-tartrate enzyme from *Rhodopseudomonas sphaeroides* does not appear to involve Fe^{2+} and is more stable [7]. However, a D-(−)-tartrate dehydratase isolated from another *Pseudomonas* species was optimally active when iron was present in the assay [8]. This paper describes a simple isolation procedure for L-(+)-tartrate dehydratase from *P. putida*, and demonstrates that it contains irons and labile sulphur in approximately equal proportions.

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2. MATERIALS AND METHODS

L-(+)-Tartrate, tartronate and biochemical reagents were obtained from Sigma. Dye-ligand columns were as described in [9]; approx. 20 were screened for their ability to bind the enzyme.

P. putida PPN1 (ATCC 12633) was grown in a defined medium [10] and a solution of trace elements, using citrate as carbon source. Actively growing cells were then transferred to medium with L-(+)-tartrate replacing the citrate to provide a 1 l starter culture. 10–15 l of medium was used to grow cells at 28°C with aeration for 20 h. Approx. 2 g cells per l was recovered on centrifuga-

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tion. Cells were extracted using a French press into a medium consisting of 30 mM K phosphate, pH 7.0, plus 5 mM succinate, 5% sucrose, 0.1 mM Fe^{2+}, 5 mM cysteine, 5 mM glutathione, 10 mM ascorbate, and 5 mM $MgCl_2$.

Z. mobilis 6-phosphogluconate dehydratase was isolated as in [5].

Samples for iron and sulphur analysis were passed through small columns of Sephadex G-25 and/or dialysed extensively against iron-free buffers. Iron analyses were carried out using a Varian AA-275 atomic absorption spectrophotometer. Acid-labile sulphide was measured using the method of Beinert [11]. Enzyme activity was determined as described [6]; units of activity are in $\mu mol \cdot min^{-1}$ at 25°C.

3. RESULTS

3.1. *Stability of enzyme*

Hurlbert and Jakoby [6] showed that extracts made in 0.01 M Tris-chloride, pH 7.0, + 5 mM glutathione and 5 mM cysteine lost tartrate dehydratase activity, but could be reactivated 100-fold by incubation with Fe^{2+}, cysteine and glutathione. We confirmed that this was so, and if the extract was made in an iron-sulphydryl buffer, the enzyme was obtained in an already active form. Manipulations on dye-ligand columns even in the presence of iron and sulphydryl reagents led to rapid and mostly irrecoverable loss of activity. A series of stability studies with a variety of agents was carried out. The presence of sucrose and/or succinate successfully stabilized the enzyme. Consequently all extracts and buffers used in purification contained 5% sucrose and 5 mM succinate.

3.2. *Purification*

After screening a number of small dye-ligand columns of closely equivalent protein-binding abilities, Procion red H-8BN was chosen as the 'negative' and Procion yellow H-E3G as the 'positive' absorbents [9]. Columns of dimensions 4 cm^2 × 6 cm were packed and equilibrated with pH 7.0 buffer. Extract containing 250–400 units tartrate dehydratase was passed through the two columns in series, and the Procion yellow H-E3G column was then worked up for the enzyme. After washing with 160 ml extraction buffer, tartrate dehydratase was eluted by including 2 mM tartronate, a strong inhibitor, in the buffer. The eluted enzyme was immediately concentrated to 1–2 ml by ultrafiltration and the sample stored frozen at −80°C. A summary of the procedure is given in table 1.

The preparations on polyacrylamide-SDS gels showed two equally strong bands at 23 and 27 kDa, with occasionally some traces of impurity at 45 kDa. Assuming that these two bands both represent the enzyme (see below), the preparation was >95% pure, with an overall recovery of at least 50%. The whole procedure, in concept and in performance is similar to that used for 6-phosphogluconate dehydratase [5].

3.3. *Structural and kinetic properties*

The enzyme was analysed for iron and acid-labile sulphide [11] after passage through a small column of Sephadex G-25 to remove excess Fe^{2+} from the buffer. Samples were also extensively dialysed against iron-free buffer, but these lost all enzymic activity. The results of several analyses are shown in table 2.

Although the expectation was that up to 8 irons

Table 1

Purification of L-(+)-tartrate dehydratase from 7.5 g (wet wt) *P. putida*

	Total protein (mg)	Total activity (units)	Specific activity ($units \cdot mg^{-1}$)
Extract	320	340	1.1
Not adsorbed on red H-8BN	225	340	1.5
Not adsorbed on yellow H-E3G	165	<30	–
Eluted from yellow H-E3G with tartronate	2.7	210	78

Table 2

Iron and acid-labile sulphide content of *P. putida* tartrate dehydratase and *Z. mobilis* 6-phosphogluconate dehydratase

Sample	μmol Fe per μmol enzyme	μmol S per μmol enzyme
Tartrate dehydratase (4 preparations)	2–4 (average 3.5)	2–6 (average 4)
6-Phosphogluconate dehydratase (2 preparations)	6–8	6–8

The molecular mass of tartrate dehydratase was taken as 100 kDa, and of 6-phosphogluconate dehydratase (a dimer) as 126 kDa

and sulphide could be found per molecule of 100 kDa, analyses mostly gave much lower values, suggesting either only one Fe_4S_4 cluster per $\alpha_2\beta_2$ structure, or possibly two clusters of Fe_2S_2. Analysis on fully active enzyme was not possible, since the removal of excess Fe^{2+} from the buffer resulted in irreversible loss of activity.

Attempts to separate the two components indicated by polyacrylamide-SDS gel electrophoresis using ion-exchange chromatography or gel filtration were unsuccessful. Gel filtration on Sephacryl S-200 in a buffer containing iron and sulphydryl compounds gave a single peak corresponding to 100 kDa. However, this could have been two tetrameric proteins of 92 and 104 kDa; samples were taken across the peak, concentrated appropriately and run on electrophoresis. The gels were then scanned with a densitometer to detect any variation in the relative proportions of the two subunits. All fractions gave close to 1 : 1 ratios for the two bands. It is concluded that there was only one protein component, of 100 kDa made up of 2 subunits of 23 kDa and 2 of 27 kDa.

4. DISCUSSION

The occurrence of iron-sulphur clusters in (de)hydratases of EC sub-class 4.2.1.x appears to be a common phenomenon, although the behavior of the enzymes in terms of both stability and required reactivation procedures differs considerably. Thus aconitase is relatively stable and can be readily reactivated to an Fe_4S_4 form; the final, labile Fe appears to be necessary for substrate binding [2]. Other dehydratases have not been studied in such detail partly because they are more labile. The *P. putida* L-(+)-tartrate dehydratase behaves in a similar fashion to the other enzymes in losing activity in the absence of excess Fe^{2+} plus reducing agents; the isolated enzyme contains structural iron and acid-labile sulphide. However, quantitation of these elements was difficult as it could only be carried out on partially inactive enzyme which may have lost part of its iron-sulphur cluster. The enzyme has a complex structure with two subunits each of 23 and 27 kDa; it would be expected by symmetry that there would be two, if not four Fe-S clusters. Our measurements have indicated rather low Fe and S contents, suggesting either only one Fe_4S_4 per tetrameric molecule, or alternatively two FeS clusters of smaller size. These possibilities could be tested using ESR.

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REFERENCES

[1] Ramsay, R.R. and Singer, T.P. (1984) Biochem. J. 221, 489–497.
[2] Johnson, M.K., Thomson, A.J., Richards, A.J.M., Peterson, J., Robinson, A.E., Ramsay, R.R. and Singer, T.P. (1984) J. Biol. Chem. 259, 2274–2282.
[3] Schloss, J.V., Emptage, M.K. and Cleland, W.W. (1984) Biochemistry 23, 4572–4580.
[4] Dreyer, J.-L. (1985) Eur. J. Biochem. 150, 145–154.
[5] Scopes, R.K. and Griffiths-Smith, K. (1984) Anal. Biochem. 136, 530–534.
[6] Hurlbert, R.E. and Jakoby, W.B. (1965) J. Biol. Chem. 240, 2772–2777.
[7] Rode, H. and Giffhorn, F. (1982) J. Bacteriol. 150, 1061–1068.
[8] Rode, H. and Giffhorn, F. (1982) J. Bacteriol. 151, 1602–1604.
[9] Scopes, R.K. (1984) Anal. Biochem. 136, 525–529.
[10] Vogel, H.J. and Bonner, D.M. (1956) J. Biol. Chem. 218, 97–106.
[11] Beinert, H. (1983) Anal. Biochem. 131, 373–378.