Purification and characterization of pyruvate:ferredoxin oxidoreductase from the anaerobic protozoon *Trichomonas vaginalis*

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The pyruvate:ferredoxin oxidoreductase from the anaerobic protozoon *Trichomonas vaginalis* is an extrinsic protein bound to the hydrogenosomal membrane. It has been solubilized and purified to homogeneity, principally by salting-out chromatography on Sepharose 4B. Low recoveries of active enzyme were caused by inactivation by O₂ and the irreversible loss of thiamin pyrophosphate. It is a dimeric enzyme of overall *M_r* 240 000 and subunit *M_r* 120 000. The enzyme contains, per mol of dimer, 7.3 ± 0.3 mol of iron and 5.9 ± 0.9 mol of acid-labile sulphur, suggesting the presence of two [4Fe–4S] centres, and 0.47 mol of thiamin pyrophosphate. The absorption spectrum of the enzyme is characteristic of a non-haem iron protein. The pyruvate:ferredoxin oxidoreductase from *T. vaginalis* is therefore broadly similar to the 2-oxo acid:ferredoxin (flavodoxin) oxidoreductases purified from bacterial sources, except that it is membrane-bound.

INTRODUCTION

In the anaerobic protozoa *Trichomonas vaginalis* and *Trichomonas foetus*, the enzymes that catalyse the fermentation of pyruvate to acetate are all localized in an unusual type of redox organelle termed a 'hydrogenosome' (Lindmark & Müller, 1973; Müller, 1980; Steinbuchel & Müller, 1986). Substrate-level phosphorylation by this pathway is thought to contribute significantly to the organism's viability (Müller, 1982).

Pyruvate:ferredoxin oxidoreductase (pyruvate synthase, EC 1.2.7.1), the first enzyme in the pathway, catalyses the oxidative decarboxylation of pyruvate in a CoA-dependent reaction yielding acetyl-CoA and CO₂. The oxidant *in vivo* is thought to be ferredoxin. The anti-trichomonal drug metronidazole can act as an alternative reductant, and this process is assumed to play an important role in its mechanism of action (Yarlett *et al.*, 1985).

Apparently analogous enzymes have been purified from *Clostridium acidi-urici* (Uyeda & Rabinowitz, 1971) and from *Halobacterium halobium* (Kerscher & Oesterhelt, 1981a) and shown to contain thiamin pyrophosphate and iron-sulphur clusters. Pyruvate:ferredoxin oxidoreductase and the closely related pyruvate:flavodoxin oxidoreductase have also been detected in a wide range of bacteria and in some other anaerobic protozoa and fungi (Buchanan, 1972; Gehring & Arnon, 1972; Reeves *et al.*, 1977; Lindmark, 1980; Weinbach *et al.*, 1980; Blaschkowski *et al.*, 1982; Neuer & Bothe, 1982; Kerscher & Oesterhelt, 1982; Shah *et al.*, 1983; Yarlett *et al.*, 1983, 1986). However, because of the O₂-induced lability of many of these enzymes, they are difficult to isolate and have not been extensively studied. The present paper reports the first purification to homogeneity of pyruvate:ferredoxin oxidoreductase from an anaerobic protozoon, and its subsequent characterization.

EXPERIMENTAL

Materials

Malate dehydrogenase (from pig heart), citrate synthase (from pig heart) and glucose oxidase (from *Aspergillus niger*), ferredoxin (from *Clostridium pasteurianum*), 2-oxo acids, coenzymes and proteins used as *M_r* markers in electrophoresis were obtained from Sigma Chemical Co., Poole, Dorset, U.K. The Bradford (1976) reagent for protein assays was from Bio-Rad Laboratories, Watford, Herts., U.K. The TSK 3000SWG and TSK 2000SWG gel-filtration columns were supplied by Anachem, Luton, Beds., U.K. Ferredoxin was purified from *T. vaginalis* as described by Gorrell *et al.* (1984), except that the final gel-filtration step using Sephadex G-75 was replaced by size-exclusion chromatography on a column (600 mm × 7 mm) of TSK 2000SWG.

Organism and growth of cells

Trichomonas vaginalis Bushby strain was grown at 37 °C in complex medium (Linstead, 1981) supplemented with 10% (v/v) heat-inactivated horse serum and 360 μM-FeSO₄ to maintain optimal concentrations for iron-sulphur proteins (Gorrell, 1985). After 48 h, cultures were harvested in late exponential phase (1.5 million cells/ml) by centrifugation at 1200 *g* for 15 min. The cells were resuspended and washed in 85 mM-NaCl/4.6 mM-KCl/1.1 mM-MgSO₄/222 mM-Na₂HPO₄/HCl buffer, pH 7.4, containing 2 g of glucose/litre. The cell pellet could be stored at –20 °C for several months without loss of pyruvate:ferredoxin oxidoreductase activity.

Determination of protein, cofactors and acetyl-CoA

Protein was generally determined by the Coomassie Blue dye-binding method (Bradford, 1976), calibrated with γ-globulin. Samples used for analyses of iron,

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sulphide and thiamin pyrophosphate were also assayed for protein by amino acid analysis. Acetyl-CoA was assayed by using malate dehydrogenase and citrate synthase (Decker, 1985). Iron was determined as described by Lovenberg *et al.* (1963) and sulphide as described by Rabinowitz (1978). Samples of ferredoxin from *Clostridium pasteurianum* were analysed as controls of these procedures and gave results in accordance with published values (Lovenberg *et al.*, 1963). Thiamin pyrophosphate was released by treatment with 0.3 M-HClO₄ and determined fluorimetrically after oxidation to thiochrome pyrophosphate (Penttinen, 1979). Total flavin was determined as outlined by Koziol (1971).

Enzyme assays

Decarboxylation of pyruvate was measured spectrophotometrically by using Methyl Viologen as the electron acceptor, essentially as described by Lindmark & Müller (1973), except that glucose, glucose oxidase and catalase were added to scavenge O₂. The assay mixture contained 100 mM-potassium phosphate buffer, pH 7.0, 2.5 mM-sodium pyruvate, 20 mM-dithiothreitol, 0.25 mM-CoA, 20 mM-Methyl Viologen, 0.1% Triton X-100, 20 mM-glucose, 10 units of glucose oxidase/ml, 10 units of catalase/ml and enzyme sample in a volume of 1 ml at 30 °C. The mixture was covered with water-saturated n-butanol to exclude O₂. The presence of the O₂-scavenging system in all assays greatly diminished the lag phase which was present. Enzyme activities were measured from the linear steady-state rates obtained after any lag phase. This rate was independent of the extent of the lag phase and was proportional to the enzyme concentration over a 1000-fold range. The ϵ_{600} for Methyl Viologen was taken as 13000 M⁻¹·cm⁻¹ (Thorneley, 1974).

Succinyl-CoA synthetase (ADP-utilizing) was assayed as described by Cha (1969). Hydrogenase was assayed as described by Lindmark & Müller (1973); again an O₂-scavenging system was included.

One unit of enzyme activity was defined as the amount of enzyme which gave a rate of 1 μ mol of product formed/min under the conditions specified.

Purification of pyruvate:ferredoxin oxidoreductase from *T. vaginalis*

All operations were carried out at 0–4 °C. All buffers were degassed, sparged with O₂-free N₂ and supplemented with 2 mM-dithiothreitol and 1 mM-Na₂S₂O₄ just before use.

(1) Preparation of membrane fraction. *T. vaginalis* cells (20 g wet wt.) were thawed, and 20 ml of 50 mM-potassium phosphate buffer, pH 7, containing 2 mM-EDTA, was added. The mixture was homogenized with 20 passes in a motor-driven Potter–Elvehjem homogenizer at 5000 rev./min. The homogenate was centrifuged at 1500 g for 10 min. Cell membranes were collected by centrifugation of the supernatant at 124 000 g for 1 h. The membranes were washed once with 15 ml of 10 mM-potassium phosphate (pH 7.4)/1 mM-EDTA/0.1 mM-sodium acetate/30% (v/v) ethanediol buffer, pH 7.4. At this stage membranes could be stored at –20 °C for months without loss of pyruvate:ferredoxin oxidoreductase activity.

(2) Salt wash of membranes. The membrane pellet was resuspended in 15 ml of 10 mM-potassium phosphate/1 mM-EDTA/1.0 M-sodium acetate buffer, pH 7.4, and stirred for 30 min. After centrifugation at 124000 g for 30 min, the tube contained a golden supernatant and a grey pellet.

(3) Salting-out chromatography. Saturated (NH₄)₂SO₄, adjusted to pH 7.4 with Tris, was added to the supernatant to give an 80%-saturated solution and stirred for 1 h. After centrifugation at 124000 g for 1 h, the pellet was resuspended in 4 ml of 2.4 M-(NH₄)₂SO₄, pH 7.4, and applied at a flow rate of 10 ml/h to a Sepharose 4B column [10 cm × 2 cm, equilibrated with 2.4 M-(NH₄)₂SO₄, pH 7.4]. The column was washed with 60 ml of equilibration buffer. Elution was carried out with a 200 ml descending gradient of (NH₄)₂SO₄ from 2.2 M to 1 M. The active fractions of the eluate were pooled and concentrated in an Amicon ultrafiltration cell fitted with a PM10 membrane. In the same cell the buffer was changed to 20 mM-Tris/HCl (pH 7.4)/0.4 M-KCl/1 mM-EDTA.

(4) Gel filtration. This was carried out with a column (600 mm × 7 mm) of TSK 3000SWG on an LKB liquid chromatograph equilibrated with 20 mM-Tris/HCl (pH 7.4)/0.4 M-KCl/1 mM-EDTA buffer. Three runs were performed, in each of which a 0.5 ml sample was applied and eluted with the same buffer at a flow rate of 0.5 ml/min. Active fractions were pooled and concentrated as before. Purified enzyme was stored in liquid N₂.

Spectra

Absorption spectra of purified pyruvate:ferredoxin oxidoreductase were recorded on a Perkin–Elmer Lambda 7 spectrophotometer.

Polyacrylamide-gel electrophoresis

Gel electrophoresis in the presence of SDS was performed on Tris/glycine-buffered 10 or 15%-(w/v)-polyacrylamide slab gels (Anderson *et al.*, 1973). Gels were stained for protein with Coomassie Brilliant Blue G-250.

Amino acid analysis

This was performed essentially as described by Perham (1978).

Ultracentrifugation

Sedimentation-equilibrium analysis was conducted at 20 °C in a Beckman model E analytical centrifuge fitted with Rayleigh interference optics, the procedures described by Nureddin & Johnson (1977) being used. Before use, the protein solution (1 mg/ml) was dialysed against 20 mM-Tris/HCl (pH 7.4)/0.4 M-KCl/1 mM-EDTA/2 mM-dithiothreitol/1 mM-Na₂S₂O₄ (I 0.42).

RESULTS

Localization and solubilization of pyruvate:ferredoxin oxidoreductase

Subcellular fractionation of trichomonads in iso-osmotic media demonstrated that pyruvate:ferredoxin oxidoreductase is present solely in their hydrogenosomes

Table 1. Summary of the purification of *T. vaginalis* pyruvate:ferredoxin oxidoreductase

For full experimental details, see the text.

Step	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Cellular extract*	20	1310	992	0.76	100	1
(1) Membrane fraction	20	554	1100	2.0	110	2.6
(2) Salt-wash supernatant	16	82	448	5.5	45	7.2
(3a) (NH ₄) ₂ SO ₄ pellet	4	25	164	6.6	17	8.6
(3b) Concentrated salting-out peak	1.5	4.5	33	7.3	3.3	9.6
(4) Concentrated TSK peak	1.2	1.1	18	18.3	1.8	24

* From 20 g wet wt. of *T. vaginalis* cells.

Fig. 1. Purification of pyruvate:ferredoxin oxidoreductase from *T. vaginalis*

Electrophoresis was carried out on a 15% polyacrylamide gel in the presence of SDS. Lane 1, membrane pellet after 0.1 M-sodium acetate salt wash (10 µg of protein); lane 2, 1.0 M-salt-wash supernatant (5 µg); lane 3, concentrated salting-out peak (10 µg); lane 4, concentrated TSK peak (5 µg).

and that the activity showed a latency revealed by disruption of the hydrogenosomal membrane, e.g. with Triton X-100 (Lindmark & Müller, 1973; Lindmark *et al.*, 1975). We found that vigorous homogenization of

T. vaginalis cells, in the absence of osmotic support, allowed measurement of most of the pyruvate:ferredoxin oxidoreductase activity, even in the absence of Triton X-100, and that all of this activity is sedimented at 124000 g for 1 h (Table 1). In contrast, the hydrogenosomal enzyme succinyl-CoA synthetase (ADP-utilizing), was not sedimented under these conditions (P. N. Lowe, unpublished work). Thus the pyruvate:ferredoxin oxidoreductase is bound to the hydrogenosomal membrane.

A range of methods were tried in order to establish a reliable method for the solubilization of pyruvate:ferredoxin oxidoreductase, using as a criterion of solubilization the appearance of activity in the supernatant after centrifugation at 124000 g for 1 h. Salt (1 M-KCl or 1 M-sodium acetate) or ionic detergents [1% (w/v) deoxycholate or 1% (w/v) cetyltrimmonium bromide] were found to be effective solubilizing agents. However, non-ionic detergents [1% (v/v) Triton X-100 or 1% (w/v) n-octyl glucoside) did not solubilize the enzyme (see the Discussion section). Sodium acetate was finally chosen so that the use of detergents could be avoided.

Dialysis of the salt-solubilized enzyme against salt-free buffer resulted in aggregation and precipitation of the enzyme protein. However, most of the activity could be regained by redissolving the precipitate in buffer containing 0.4 M-KCl. Therefore, for the purification of the solubilized enzyme, high salt ($I \geq 0.4$) was maintained in all buffers.

Purification and stability of pyruvate:ferredoxin oxidoreductase

The purification of pyruvate:ferredoxin oxidoreductase is summarized in Table 1. The recovery was typically 1–3%, representing an apparent purification of 15–20-fold over the cellular extract. The final specific activity was about 10–20 units/mg of protein. Fig. 1 shows fractions throughout the purification, analysed by SDS/polyacrylamide-gel electrophoresis. No advantage was found in starting from hydrogenosome-enriched fractions (Lindmark & Müller, 1973) rather than whole-cell extracts. The inclusion of ethanediol in the low-salt washing buffer increased the amount of other proteins removed in this step. Typically, about half of the membrane-associated pyruvate:ferredoxin oxidoreductase activity is released by the first salt extraction. The yield was only marginally improved by re-extraction.

Salting-out chromatography on Sepharose 4B afforded excellent purification (Fig. 2) at the expense of a poor recovery of enzyme activity (< 20%). It can be seen by comparison of Fig. 2 with Table 1 that the specific activity of the enzyme was only marginally increased by salting-out chromatography, although the purity was greatly increased. We believe that this is due to an irreversible loss of enzyme activity, as a result of the long run time (20 h) necessary to achieve optimal resolution. The degree of purification shown in Table 1 is thus likely to be an underestimate. The salt extraction also released hydrogenase activity from the membrane, but it was eluted ahead of pyruvate:ferredoxin oxidoreductase and well separated from it. Yields of activity were not improved by including thiamin pyrophosphate/Mg²⁺,

FeSO₄ or antipain [a potent inhibitor of trichomonal proteinases (Coombs & North, 1983)] in the buffers.

From 1000–1500 mg of starting protein, 1–1.5 mg of pure pyruvate:ferredoxin oxidoreductase was obtained, which indicates that it represents at least 0.1% of total cell protein. It has been estimated that hydrogenosomes represent 5–10% of *T. vaginalis* cell protein (Lindmark *et al.*, 1975). Thus the enzyme represents at least 1% of the protein in the organelle.

Solubilized pyruvate:ferredoxin oxidoreductase activity was considerably more labile than that of the membrane-bound enzyme. Incubation of solubilized enzyme at 4 °C at pH 7.4 resulted in the irreversible loss of about 20% of activity after 5 h and 80% after 22 h. Maintenance of strict anaerobic conditions using di-

Fig. 2. Purification of pyruvate:ferredoxin oxidoreductase by salting-out chromatography

(a) (NH₄)₂SO₄ pellet (60 units, 3 ml) was applied to a Sepharose 4B column (10 cm × 2 cm) equilibrated with 2.4 M-(NH₄)₂SO₄ and eluted as described in the Experimental section. Column fractions (3.5 ml) were assayed for protein (—, A₂₈₀) and pyruvate:ferredoxin oxidoreductase (○) activity. ----, [(NH₄)₂SO₄]. (b) SDS/polyacrylamide-gel electrophoresis of column fractions.

Table 2. Molecular properties of purified pyruvate:ferredoxin (flavodoxin) oxidoreductases

Cofactors were analysed as described in the text. Abbreviation: ND, not determined.

Molecular property	<i>Trichomonas vaginalis</i>	<i>Clostridium aci-di-urici</i>	<i>Halobacterium halobium</i>	<i>Klebsiella pneumoniae</i> *
M_r	240 000	240 000†	256 000	240 000‡
Subunits/ M_r	$2 \times 120 000$	ND	$2 \times 86 000$, $2 \times 42 000$	$2 \times 120 000$
Fe ²⁺ (mol/mol)	7.3 ± 0.3 ($n = 4$)	6	7.6	8.0 ± 0.6
S ²⁻ (mol/mol)	5.9 ± 0.9 ($n = 3$)	3	5.2	6.6 ± 0.2
TPP (mol/mol)	0.47	0.5–0.8	1.3	ND
Flavin (mol/mol)	0.005	Trace	< 0.005	ND
Reference . . .	The present study	Uyeda & Rabinowitz (1971)	Kerscher & Oesterhelt (1981)	Shah <i>et al.</i> (1983)

* Pyruvate:flavodoxin oxidoreductase.

† The M_r was estimated by Uyeda & Rabinowitz (1971) from the sedimentation coefficient ($s_{20,w}^0$) by assuming a diffusion coefficient ($D_{20,w}^0$) of 3.6×10^{-7} and a partial specific volume ($\bar{v}$) of 0.72 ml/g, and thus might be unreliable.‡ The M_r of partially purified pyruvate:flavodoxin oxidoreductase from *E. coli* has an M_r of 200 000 (Blaschkowski *et al.*, 1982). **Fig. 3. U.v. and visible absorption spectrum**

The spectrum of pure pyruvate:ferredoxin oxidoreductase (1.3 mg of protein/ml), dissolved in 20 mM-Tris/HCl (pH 7.4)/0.4 M-KCl/1 mM-EDTA, was recorded in a cuvette of pathlength 1 cm.

thionite and dithiothreitol decreased the rate of inactivation. Inclusion of phospholipids or glycerol did not prevent inactivation at 4 °C. Enzyme activity was stable for several weeks after storage in liquid N₂.

Homogeneity and M_r

Purified pyruvate:ferredoxin oxidoreductase was essentially homogeneous, as judged by polyacrylamide-gel electrophoresis in the presence of SDS (Fig. 1). From comparison with mobilities of standard proteins, its

apparent subunit M_r was estimated as 120 000 on Tris/glycine-buffered gels and 127 000 on sodium phosphate-buffered gels. By using the partial specific volume of 0.741 ml/g calculated (Lee & Timasheff, 1979) from its amino acid composition (Table 3 below), sedimentation-equilibrium analysis of the native enzyme gave an estimate of the weight-average M_r as 240 000. An independent estimate of M_r of the native enzyme was obtained by gel filtration on a TSK 3000SWG column. A good linear correlation between $\log M_r$ and K_{av} for standard proteins was obtained (results not shown). Pyruvate:ferredoxin oxidoreductase was eluted as a broad peak, with an apparent M_r of about 275 000.

Chemical composition

In common with the other 2-oxoacid:ferredoxin oxidoreductases characterized so far, the enzyme from *T. vaginalis* contains non-haem iron and acid-labile sulphur (Table 2). The absorption spectrum of purified *T. vaginalis* pyruvate:ferredoxin oxidoreductase exhibited a broad absorption band of low intensity between 350 and 500 nm (Fig. 3) characteristic of iron-sulphur proteins. Thiamin pyrophosphate in sub-stoichiometric amounts, but no flavin nucleotide, was detected (Table 2). The low value found for thiamin pyrophosphate suggests losses during purification, since control samples of thiamin pyrophosphate could be analysed with excellent recovery. However, addition of thiamin pyrophosphate to the assay mixture did not increase the activity of the enzyme, suggesting this loss must be irreversible. The amino acid composition of the enzyme is shown in Table 3.

Catalytic properties

A steady-state kinetic analysis of the purified *T. vaginalis* enzyme gave a K_m for pyruvate of 0.14 mM and a K_m for CoA of 2.5 μ M. The enzyme obeyed Bi Bi Ping Pong kinetics (Fig. 4a), except at very high CoA concentrations, where substrate inhibition occurred (Fig. 4b). The inhibition produced by the product, acetyl-CoA, in the presence of saturating CoA, was competitive with respect to pyruvate (Fig. 4c), with $K_i = 100 \pm 20 \mu$ M. The

kinetic parameters of crude membrane preparations (K_m for pyruvate, 0.13 mM; K_m for CoA, 3.5 μ M) were identical, within experimental error, with those for the pure solubilized enzyme.

Pure enzyme accepted 2-oxobutyrate, pyruvate or 2-oxoglutarate as substrates in the standard assay, with

Table 3. Amino acid composition of *T. vaginalis* pyruvate:ferredoxin oxidoreductase

The results (means $\pm$ s.d.) were obtained from duplicate analyses of 24, 48 and 96 h hydrolysates. The values for threonine and serine were obtained by linear extrapolation to zero hydrolysis time. Isoleucine and valine were taken from 96 h hydrolysates. Cysteine was determined, after performic acid oxidation, as cysteic acid.

Amino acid	Composition (mol/mol of protein)
Cys	24
Asx	196 $\pm$ 8
Thr	110 $\pm$ 2
Ser	187 $\pm$ 7
Glx	258 $\pm$ 6
Pro	132 $\pm$ 12
Gly	289 $\pm$ 13
Ala	182 $\pm$ 14
Val	111 $\pm$ 1
Met	33
Ile	100 $\pm$ 9
Leu	157 $\pm$ 6
Tyr	80 $\pm$ 3
Phe	75 $\pm$ 8
His	63 $\pm$ 6
Trp	35*
Lys	136 $\pm$ 7
Arg	101 $\pm$ 1

* Determined spectroscopically (Edelhoch, 1967).

K_m^{app} values of 0.1 mM, 0.14 mM and 0.5 mM respectively, and $V_{\text{max}}^{\text{app}}$ values relative to pyruvate of 0.45, 1.00 and 0.05 respectively. The stoichiometry of the reaction was deduced by assay of the acetyl-CoA formed. The results (Table 4) show that, in the presence of saturating pyruvate and Methyl Viologen, the reaction yields 1 mol of acetyl-CoA for every mol of CoA used. To test its specificity for electron acceptor, pyruvate:ferredoxin oxidoreductase was assayed with a variety of low-potential electron acceptors (Table 5). The enzyme was unable to use NAD⁺ or NADP⁺, but was able to utilize the artificial compounds, Methyl Viologen and Benzyl Viologen, and either bacterial, or protozoal, ferredoxin.

DISCUSSION

Pyruvate:ferredoxin oxidoreductase has been recognized for a number of years to be an important enzyme in trichomonal energy metabolism, but its purification has been hampered by its instability to O₂ (Lindmark & Müller, 1973; Lindmark *et al.*, 1975). An additional problem we encountered stemmed from our observation that the enzyme is bound to the hydrogenosomal membrane; although it can be largely released by washing with buffers of high ionic strength, subsequent decrease in *I* to below 0.4 resulted in aggregation of the protein, and this limited the choice of purification techniques that could be used. Salting-out chromatography, using a method similar to that used to purify halophilic enzymes from *Halobacterium halobium* (Mevarech *et al.*, 1976), was a very effective technique, but the time required for this step unavoidably resulted in low recovery of enzyme activity. In this method proteins are adsorbed from solutions of high salt concentration on to agarose gels and resolved by a decreasing salt gradient. The loss of activity (Table 1) during this step was largely accounted for by the lability of the enzyme, and thus does not represent a poor recovery of enzyme protein.

A distinctive feature of the *T. vaginalis* pyruvate:

Fig. 4. Kinetic analysis of pyruvate:ferredoxin oxidoreductase

(a) Double-reciprocal plot of initial velocity versus pyruvate concentration at various fixed concentrations of CoA: 0.5 μ M (▲); 1.0 μ M (●); 2.5 μ M (□); 5.0 μ M (△); 10 μ M (○). Citrate synthase and oxaloacetic acid were included to regenerate CoA so as to maintain a constant concentration during the assay. (b) Double-reciprocal plot of initial velocity versus CoA concentration with a saturating concentration of pyruvate (2.5 mM). (c) Double-reciprocal plot of initial velocity versus pyruvate concentration with a saturating concentration of CoA (0.25 mM) with various concentrations of acetyl-CoA: 50 μ M (○); 100 μ M (▲); 200 μ M (■); 400 μ M (●).

Table 4. Stoichiometry of the forward reaction catalysed by pyruvate:ferredoxin oxidoreductase

Assay	CoA added (μmol)	Methyl Viologen reduced (μmol)	CoA used* (μmol)	Acetyl-CoA formed (μmol)
1	39	84.6	42.3	40.5
2	29.3	53.8	26.9	29.0
3	19.5	29.2	14.6	18.5

* Assuming 2 Methyl Viologen molecules react with 1 molecule of CoA.

Table 5. Activities of pyruvate:ferredoxin oxidoreductase with different electron acceptors

Activities were calculated using the following absorption coefficients ($\text{M}^{-1}\cdot\text{cm}^{-1}$): Methyl Viologen, $\epsilon_{600} = 13000$ (Thorneley, 1974); Benzyl Viologen, $\epsilon_{555} = 7550$ (Sim & Vignais, 1978); $\text{NAD}^+/\text{NADP}^+$, $\epsilon_{340} = 6220$; ferredoxin, $\epsilon_{390} = 17300$ (Lindmark & Müller, 1973). The 2-oxo acid substrate was pyruvate in each case.

Electron acceptor	Specific activity (units/mg of protein)
Methyl Viologen (20 mM)	2.15
Benzyl Viologen (20 mM)	2.00
Clostridial ferredoxin (0.5 mg/ml)	1.50
<i>T. vaginalis</i> ferredoxin (0.2 mg/ml)	0.60
NAD^+ or NADP^+ (5 mM)	0

ferredoxin oxidoreductase is its behaviour as an extrinsic membrane protein, in that it can be solubilized under mild conditions at high ionic strength. Although ionic detergents also solubilized the enzyme, non-ionic detergents did not. We believe that this is because the enzyme aggregates in the presence of Triton X-100. This idea is supported by the observation that salt-solubilized pyruvate:ferredoxin oxidoreductase, when brought out of solution by dialysis against salt-free buffer, could be re-dissolved in either 0.4 M-KCl or 0.1% deoxycholate, but not in 1% Triton X-100.

Pyruvate:ferredoxin (flavodoxin) oxidoreductases from *Clostridium*, *Halobacterium*, *Klebsiella*, *Escherichia coli*, and non-trichomonal protozoa, e.g. *Entamoeba histolytica*, differ from that from of *T. vaginalis* in that the enzymes are not apparently membrane-bound. However, the *Halobacterium* enzyme was only active in the presence of high salt concentrations, and thus, if it has similar properties to the enzyme from *T. vaginalis*, these might have been overlooked.

The value of 240000 for the weight-average M_r is very similar to those values determined by comparable methods for other pyruvate:ferredoxin oxidoreductases and the related enzymes that use flavodoxin instead of ferredoxin (Table 2). The M_r of the enzyme from *C. acidi-urici* reported by Uyeda & Rabinowitz (1971) is only an estimate based on the measurement of its sedimentation coefficient, with no experimental data on partial specific volume, diffusion coefficient or molecular shape, and thus might be unreliable. A value of 120000 for the M_r of the subunit fits well with the suggestion that the

trichomonal enzyme is a homodimer as isolated. A value of 240000 for the M_r was used when calculating cofactor content and amino acid content per mol of enzyme. The homodimeric subunit composition agrees closely with that of the pyruvate:flavodoxin oxidoreductase from *Klebsiella pneumoniae* (Table 2), but differs from the enzyme from *H. halobium*, the only other similar enzyme with a known subunit structure. The latter enzyme has an $\alpha_2\beta_2$ structure; it is noteworthy that the sum of the M_r values of the α - and β -subunits (128000) is very similar to the individual subunit M_r of the *T. vaginalis* and *K. pneumoniae* enzymes.

Pyruvate:ferredoxin oxidoreductase from *T. vaginalis* is similar to equivalent bacterial enzymes (Table 2 and Fig. 3) in being a thiamin pyrophosphate-containing iron-sulphur protein. The instability of anaerobic oxidoreductases to O_2 has been previously noted for nitrate reductase (Carlson *et al.*, 1982), nitrogenase (Wang *et al.*, 1985), hydrogenases (e.g. Arp & Burris, 1979) and pyruvate:ferredoxin oxidoreductases [e.g. the enzyme from *Dastricha ruminantium* (Yarlett *et al.*, 1981), which is completely inactivated by even a 30 s exposure of O_2], and it is suggested that this may be due to irreversible oxidation of the iron-sulphur centres.

The sulphide content of the enzyme is likely to be an underestimate (Kerscher & Oesterhelt, 1981a). From the results of the more reliable iron determination it is likely that the enzyme contains two $[4\text{Fe}-4\text{S}]$ clusters per enzyme molecule. This would be consistent with each subunit bearing one cluster.

The thiamin pyrophosphate content of the *T. vaginalis* enzyme is slightly lower than that estimated for the clostridial enzyme and much lower than that found in the *H. halobium* enzyme. It is likely that, during the isolation of the *T. vaginalis* enzyme, an irreversible loss of thiamin pyrophosphate occurred. As with these two bacterial enzymes, addition of thiamin pyrophosphate to the purified enzyme did not result in a recovery of activity. In contrast, the *E. coli* pyruvate:flavodoxin oxidoreductase showed a total dependence upon exogenous thiamin pyrophosphate (Blaschkowski *et al.*, 1982).

The catalytic parameters of the pure solubilized enzyme are identical with those of the membrane-bound form, suggesting that the structure of the enzyme is little altered by removal from the membrane. The K_m for CoA of the *T. vaginalis* enzyme is similar to that of the *T. foetus* enzyme measured in a large-granule fraction (Lindmark & Müller, 1973), but the K_m for pyruvate of the *T. vaginalis* enzyme (0.14 mM) is much lower than that of the *T. foetus* enzyme (3.2 mM). The Ping Pong kinetics, substrate inhibition by CoA and competitive inhibition by acetyl-CoA at saturating pyruvate concentrations are consistent with the mechanism of action proposed for the enzyme from *H. halobium* (Kerscher &

Oesterhelt, 1981b), in which pyruvate decarboxylation precedes binding of CoA.

The substrate specificity of the *T. vaginalis* enzyme resembles that of the *H. halobium* enzyme (Kerscher & Oesterhelt, 1981a) in that the enzyme catalyses a rapid reaction with 2-oxobutyrate and a slow reaction with 2-oxoglutarate as well as with pyruvate. It differs markedly from the pyruvate:flavodoxin oxidoreductases from *E. coli* (Blaschkowski *et al.*, 1982) and of *K. pneumoniae* (Shah *et al.*, 1983), which show a high specificity for pyruvate.

With the exception of fungi (Yarlett *et al.*, 1986) and possibly algae, protozoa are the only eukaryotes known to possess pyruvate:ferredoxin oxidoreductase. Respiratory eukaryotes utilize the pyruvate dehydrogenase multienzyme complex, which is distinct in terms of its large size, presence of flavin nucleotide and use of NAD⁺ as oxidant. The existence in trichomonads of hydrogenase, another common prokaryotic enzyme absent from higher eukaryotes, makes the evolutionary origins of such protozoa and, in particular, the hydrogenosome, an interesting area for speculation. Interestingly, the aspartate aminotransferase from *T. vaginalis* also shows similarities with its bacterial counterparts (Lowe & Rowe, 1985). The availability of pyruvate:ferredoxin oxidoreductase from a eukaryotic source will allow comparison with its bacterial counterparts and also with the pyruvate dehydrogenase complexes. Inhibition studies will allow us to assess the role of hydrogenosomal energy metabolism for the viability of *T. vaginalis*.

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