Dihydrolipoamide dehydrogenase from *Trypanosoma brucei*

Characterization and cellular location

Michael J. DANSON,*† Kevin CONROY,* Andrew McQUATTIE† and Kenneth J. STEVENSON‡

*Department of Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, U.K., and ‡Division of Biochemistry, Department of Chemistry, University of Calgary, Calgary, Alberta, Canada T2N 1N4

Dihydrolipoamide dehydrogenase has been discovered in the bloodstream form of the eukaryotic African parasite, *Trypanosoma brucei*. The enzyme catalysed the stoichiometric oxidation of dihydrolipoamide by NAD^+ and exhibited a hyperbolic dependence of catalytic activity on the concentrations of both dihydrolipoamide and NAD^+ . Chemical modification with the tervalent arsenical reagent *p*-aminophenyldichloroarsine indicates the involvement in catalysis of a reversibly reducible disulphide bond. Plasma-membrane sheets were purified from *T. brucei*, and it was shown that virtually all the dihydrolipoamide dehydrogenase remained closely associated with this membrane preparation. *T. brucei* apparently lacks the 2-oxoacid dehydrogenase multienzyme complexes of which dihydrolipoamide dehydrogenase is usually an integral component. In the context of this absence, the possible function of trypanosomal dihydrolipoamide dehydrogenase is discussed, with particular reference to its cellular location in the plasma membrane.

INTRODUCTION

Dihydrolipoamide dehydrogenase (EC 1.8.1.4) catalyses the NAD^+ -dependent oxidation of dihydrolipoamide [reviewed by Williams (1976)]:

The enzyme fulfils this function in the pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complexes and is an integral component of each of these multienzyme structures. However, we have reported previously (Danson, 1984; Danson *et al.*, 1984, 1986) that, although archaebacteria do not possess these 2-oxo acid dehydrogenase complexes, they do have a dihydrolipoamide dehydrogenase, although its function *in vivo* remains unclear.

In the present paper we report our investigation of dihydrolipoamide dehydrogenase in the eukaryotic African parasite, *Trypanosoma brucei*. The bloodstream form of this trypanosome is totally dependent on glycolysis for ATP production, as it has no lipid or carbohydrate reserves and the mitochondrion does not possess a competent citric acid cycle (reviewed by Fairlamb, 1982). Thus it does not have the NAD -linked 2-oxo acid dehydrogenase complexes, and under aerobic conditions glucose is metabolized almost completely to pyruvate, approx. 2 mol of pyruvate being produced per mol of glucose (Fairlamb, 1982). *T. brucei* therefore provides us with a eukaryotic organism in which to look for the presence of dihydrolipoamide dehydrogenase in the absence of the 2-oxo acid dehydrogenase complexes. We not only report its presence and enzymological characteristics, but also show that it is associated with the plasma membrane of the trypanosome. The functional significance of its presence and cellular location is discussed.

EXPERIMENTAL

Materials

Chemicals used were of analytical grade or the finest grade commercially available. NAD^+ , NADP^+ and NADH were from Boehringer, Mannheim, Germany; Nbs_2 and type II-S soya-bean L- α -phosphatidylcholine were from Sigma Chemical Co., Poole, Dorset, U.K.; DL-lipoamide was from BDH Chemicals, Poole, Dorset, U.K.; and high-purity Triton X-100 was from Pierce and Warriner (U.K.), Chester, U.K. DL-Dihydrolipoamide was prepared by the reduction of DL-lipoamide with NaBH_4 (Reed *et al.*, 1958), and *p*-aminophenyldichloroarsine ($\text{H}_2\text{NPhAsCl}_2$) was synthesized as described by Stevenson *et al.* (1978).

Growth and preparation of cells

Cells of the long slender form of *T. brucei* were isolated from the blood of Wistar rats infected with 1×10^7 – 3×10^7 cells of strain MITat 1.1 by intraperitoneal injection as described by Eienthal & Panes (1985) and were further purified by chromatography on DEAE-cellulose (Lanham & Godfrey, 1970).

Cell extraction

Cells (approx. 0.1 g wet wt.) were resuspended in 1 ml of 50 mM-potassium phosphate (pH 7.0)/2 mM-EDTA. The suspension was sonicated at 0 °C for 3×30 s at 40 W with a 3 mm probe on an ultrasonic disintegrator; the cell debris was removed by centrifugation for 3 min at 10000 *g* (r_{av} 5.5 cm) and the supernatant stored at 4 °C.

Enzyme assays

Dihydrolipoamide dehydrogenase was assayed at 30 °C in 50 mM-potassium phosphate (pH 7.0)/2 mM-EDTA containing 0.4 mM-dihydrolipoamide and 1 mM- NAD^+ . The reaction, in a final volume of 1 ml, was

Abbreviation used: $\text{H}_2\text{NPhAsCl}_2$, *p*-aminophenyldichloroarsine.

† To whom all correspondence should be addressed.

started with enzyme and its progress was monitored by the increase in A_{340} .

Malate dehydrogenase was assayed at 30 °C in 50 mM-potassium phosphate (pH 7.0)/2 mM-EDTA containing 0.1 mM-NADH and 0.2 mM-oxaloacetate. The reaction, in a final volume of 1 ml, was started with enzyme and its progress was monitored by the decrease in A_{340} .

Chemical modification with $H_2NPhAsCl_2$

Modification of dihydrolipoamide dehydrogenase with 0.5 mM- $H_2NPhAsCl_2$ was carried out at 4 °C in 50 mM-potassium phosphate (pH 7.0)/2 mM-EDTA and in the presence and absence of 50 μ M-NADH.

Preparation of trypanosomal plasma membrane

Sheets of plasma membrane from *T. brucei* were prepared as described by Voorheis *et al.* (1979). In outline, this procedure was as follows. Fresh trypanosomal cells were resuspended in 10 ml of Tes buffer (2 mM-Tes, pH 7.5, containing 150 mM-KCl, 1 mM-EGTA, 1 mM-2-mercaptoethanol and 0.1 mM-phenylmethanesulphonyl fluoride). The cells were swelled by the addition of up to 30 ml of distilled water containing 0.1 mM-phenylmethanesulphonyl fluoride and 10 μ g of leupeptin/ml (4 °C) and were then homogenized in a tight-fitting glass Dounce homogenizer. Immediately after cell breakage, the ionic strength of the homogenate was restored by the addition of 2 ml of 3 M-KCl for every 40 ml of homogenate. The homogenate was then centrifuged at 7500 g_{av} for 10 s to give a supernatant (S_1) and pellet (P_1). Pellet P_1 was washed in the Tes buffer containing 10 μ g of leupeptin/ml and was then treated with DNAase as described by Voorheis *et al.* (1979). The pellet (P_2), collected by centrifugation (as above), was resuspended in 40% (w/v) sucrose in Tes buffer and centrifuged at 70000 g_{av} for 3 h on a linear 40–60% sucrose gradient in the same buffer. Plasma-membrane sheets comprised the most prominent dense band; these were removed, washed three times in Tes buffer and finally resuspended in 50 mM-potassium phosphate (pH 7.0)/2 mM-EDTA. Assays of dihydrolipoamide dehydrogenase and malate dehydrogenase were carried out on the day of the preparation.

Preparation of Triton extract

Plasma membranes were incubated with 0.5% (w/v) Triton X-100 in 10 mM-Tris/HCl buffer, pH 7.4, for 20 min at 4 °C. After centrifugation at 20000 g for 60 min, the supernatant was treated with Bio-Beads SM-2 for 16 h (0.3 g wet wt. of beads/ml) at 4 °C.

Protein determination

Protein was measured by the method of Markwell *et al.* (1981). Interference by the presence of Triton X-100 was overcome by the addition of 3% (w/v) SDS (Carruthers & Melchior, 1984).

RESULTS

Presence of dihydrolipoamide dehydrogenase in *T. brucei*

Dihydrolipoamide dehydrogenase activity was detected in cell-free extracts of *T. brucei* at a specific activity of 0.03 μ mol of NADH produced/min per mg of protein. The rate of production of NADH was directly

Fig. 1. Purification of plasma-membrane sheets of *T. brucei* and cellular location of dihydrolipoamide dehydrogenase and malate dehydrogenase

Plasma-membrane sheets from *T. brucei* were purified, and the enzymic activities of dihydrolipoamide dehydrogenase (■) and malate dehydrogenase (□) were assayed, as described in the Experimental section. The enzymic activities are expressed as a percentage of their values in the unfractionated homogenate (dihydrolipoamide dehydrogenase, 0.74 μ mol of NADH produced/min; malate dehydrogenase, 40.6 μ mol of NADH oxidized/min). (A) Unfractionated cell homogenate; (B) pellet P_1 ; (C) supernatant S_1 ; (D) final washed pellet of plasma membranes; (E) Triton X-100 extract of purified plasma membranes, after removal of detergent with Bio-Beads.

proportional to the amount of enzyme extract in the assay, and all activity was destroyed on boiling the extract for 10 min. The enzyme was specific for NAD^+ , no activity being detectable when $NADP^+$ was substituted for NAD^+ . The stoichiometry of the reaction was measured by monitoring NADH production at 340 nm and by titrating unchanged dihydrolipoamide with 5,5'-dithiobis-(2-nitrobenzoic acid); 1.06 (± 0.06) mol of NADH was produced/mol of dihydrolipoamide oxidized.

These data represent the first report of dihydrolipoamide dehydrogenase activity in a eukaryotic organism which lacks the NAD-linked 2-oxo acid dehydrogenase multienzyme complexes.

Cellular location of dihydrolipoamide dehydrogenase

Plasma-membrane sheets were prepared from *T. brucei* by the method of Voorheis *et al.* (1979) as described in the Experimental section. The enzymic activities of dihydrolipoamide dehydrogenase and of malate dehydrogenase were measured in the homogenate, supernatant (S_1) and pellet (P_1) arising from differential centrifugation of this homogenate, and in the final washed pellet of plasma membranes. The data are given in Fig. 1. In agreement with the results reported by Voorheis *et al.* (1979), the membranes were essentially free of malate dehydrogenase, less than 2% of the original enzyme activity of the homogenate being found in the final preparation. In direct contrast (Fig. 1), the dihydro-

Fig. 2. Chemical modification of *T. brucei* dihydrolipoamide dehydrogenase with $\text{H}_2\text{NPhAsCl}_2$

Purified plasma membranes of *T. brucei* were incubated at 4 °C in 50 mM-potassium phosphate (pH 7.0)/2 mM-EDTA, in the presence of 0.5 mM- $\text{H}_2\text{NPhAsCl}_2$ ($\blacktriangle$), 50 μM -NADH ($\bullet$) or 0.05 mM- $\text{H}_2\text{NPhAsCl}_2$ and 50 μM -NADH ($\circ$). Dihydrolipoamide dehydrogenase was assayed spectrophotometrically at 340 nm as described in the Experimental section.

lipoamide dehydrogenase was co-purified with the plasma membranes, with only 10% of the activity being retained in the supernatant (S_1). This observation, that the enzyme is retained with the membrane sheets through a number of purification procedures and washing steps, strongly suggests a close interaction of dihydrolipoamide dehydrogenase with trypanosomal plasma membrane.

Treatment of the purified plasma membrane with 0.5% (w/v) Triton X-100 resulted in $\geq 80\%$ of the dihydrolipoamide dehydrogenase being recovered in the supernatant after centrifugation and removal of the detergent by Bio-Beads (Fig. 1).

To provide a positive identification of the nature of the membrane in the final washed pellet, we have monitored throughout the preparation the ouabain-sensitive $\text{Na}^+ + \text{K}^+$ -stimulated ATPase, which Voorheis *et al.* (1979) find to be associated with the plasma membrane. In our hands, the activity of this enzyme is both variable and very difficult to measure in the presence of comparatively large amounts of other phosphatases. Nevertheless, we find this ATPase to be retained in pellet P_1 , and up to 60% of the homogenate activity to be in the final membranes. However, because of the difficulties we have encountered in assaying this enzyme, we would not wish to use these data alone as positive evidence for the presence of plasma membranes. Therefore we have subjected the final preparation to electron-microscopic examination. The purified membrane was observed to contain the pellicular microtubular array which Voorheis *et al.* (1979) state is a marker for 'the unequivocal identification of the isolated membrane material being derived exclusively from the plasma membrane'. On this basis, the plasma membrane in our preparation is confirmed.

Kinetic properties of dihydrolipoamide dehydrogenase

In both sonicated cell-free extracts and purified plasma membrane, the enzyme showed a hyperbolic dependence

Table 1. Kinetic properties of *T. brucei* dihydrolipoamide dehydrogenase

Sonicated cell-free extracts were prepared and plasma membranes were purified as described in the Experimental section. The K_m for dihydrolipoamide was determined at a fixed NAD^+ concentration of 1 mM; the K_m for NAD^+ was determined at a fixed dihydrolipoamide concentration of Eisenthal & Cornish-Bowden (1974). Means $\pm$ S.E.M. are shown where appropriate.

	Cell-free sonicated extract	Purified plasma membranes
K_m (dihydrolipoamide)	0.18 (± 0.04) mM	0.13 (± 0.03) mM
K_m (NAD^+)	0.35 (± 0.03) mM	0.28 (± 0.05) mM
Sp. activity ($\mu\text{mol/min per mg of protein}$)	0.029	0.229

of rate on each substrate. The data were analysed by the direct linear plot (Eisenthal & Cornish-Bowden, 1974), and the kinetic constants determined are summarized in Table 1. There were no significant differences in K_m values between the two enzyme preparations, although the specific activity in the plasma membrane was 8-fold greater than that observed in the sonicated cell-free extracts. This increase in specific activity compares with reported values of 15-fold for ouabain-sensitive $\text{Na}^+ + \text{K}^+$ -stimulated ATPase and 5-fold for adenylate cyclase (Voorheis *et al.*, 1979).

Chemical modification of dihydrolipoamide dehydrogenase with $\text{H}_2\text{NPhAsCl}_2$

The catalytic mechanism of dihydrolipoamide dehydrogenase normally involves the alternate oxidation and reduction of an intrachain disulphide bond (Williams, 1976). Thus its ligands dihydrolipoamide and NADH will render the active-site disulphide bond susceptible to dithiol-specific trivalent arsenicals (Adamson & Stevenson, 1981; Adamson *et al.*, 1984), a phenomenon which is characteristic of this enzyme and which may be used to help establish that the observed enzymic activity is the result of a true dihydrolipoamide dehydrogenase.

In accordance with the normal catalytic mechanism, the enzyme from both cell-free extracts and purified plasma membrane was rapidly inactivated by $\text{H}_2\text{NPhAsCl}_2$ in the presence of NADH (Fig. 2), whereas the reagent alone caused no significant loss of enzymic activity.

DISCUSSION

The data in the present paper constitute the first report of the presence of dihydrolipoamide dehydrogenase in *T. brucei*. The significance of this discovery stems from a number of observations. Firstly, the bloodstream form of *T. brucei* does not have the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes (Fairlamb, 1982; M. J. Danson & R. Crisp, unpublished work). In organisms which do possess these multienzyme systems, dihydrolipoamide dehydrogenase is an integral component of such complexes, and therefore its presence in

T. brucei suggests that this enzyme may have an additional metabolic role, which remains to be elucidated. This is the first time that the enzyme has been found in the absence of the 2-oxo acid dehydrogenase complexes in a eukaryotic organism, although we have previously reported a similar situation in the halophilic (Danson *et al.*, 1984, 1986), thermoacidophilic and methanogenic (Danson, 1984) archaeobacteria. We have purified dihydrolipoamide dehydrogenase from *Halobacterium halobium* (Danson *et al.*, 1986) and find it to be remarkably similar to its counterpart in non-archaeobacterial species in subunit arrangement and in its catalytic and kinetic properties. Similarly, in the present paper, we demonstrate that dihydrolipoamide dehydrogenase from *T. brucei* specifically catalyses the stoichiometric oxidation of dihydrolipoamide by NAD⁺, and we provide evidence from chemical modification studies that a reversibly reducible disulphide bond is essential to catalysis, as has been found in other dihydrolipoamide dehydrogenases (Williams, 1976).

It should be noted that Ryley (1962) found that oxidized lipoic acid increased the rate of oxidation of NADH by homogenates of *Trypanosoma rhodesiense*, indicating the presence of dihydrolipoamide dehydrogenase in this trypanosome. However, the organism can also metabolize pyruvate to CO₂ and it may be that the enzyme is functioning in its recognized role in pyruvate oxidation in *T. rhodesiense*, a situation not occurring in *T. brucei*.

A second significant factor is that *T. brucei* dihydrolipoamide dehydrogenase appears to be located specifically in the plasma membranes of the organism. Our evidence for this comes from the co-purification of the enzyme with the plasma membrane. Moreover, in preliminary experiments, we have shown, by the method of Kasahara & Hinkle (1977), that dihydrolipoamide dehydrogenase, extracted from the purified plasma membranes with Triton X-100, can be reconstituted into soya-bean L- α -phosphatidylcholine liposomes to a specific activity the same as that in the purified membranes (K. Conroy, M. J. Danson, R. Eienthal & G. Holman, unpublished work).

This is the first time that a eukaryotic dihydrolipoamide dehydrogenase has been reported to be membrane-associated, and such an attachment may suggest a function specific to that location. Indeed, evidence has been presented (Richarme, 1985; Richarme & Heine, 1986) that lipoic acid and dihydrolipoamide dehydrogenase may be involved in the *Escherichia coli* binding-protein-dependent transport of galactose and maltose. In this system, the enzyme is distinct from the *Lpd* gene product, which functions in the 2-oxo acid dehydrogenase complexes (Richarme & Heine, 1986). Additionally, it has been observed that the dithiol-specific tervalent arsenical phenylarsine oxide inactivates insulin-stimulated hexose transport by 3T3-L1 adipocytes (Frost & Lane, 1985). These authors noted that this inactivation of hexose transport had similar characteristics to the inactivation of 2-oxo acid dehydrogenases by the same arsenical (reacting with the lipoyl groups on the acetyltransferase component); however, the site of reaction of phenylarsine oxide with the adipocytes has not yet been defined.

An assay for the transport of glucose into *T. brucei* has now been developed (Game *et al.*, 1986). It should therefore be possible to determine the effects of tervalent

arsenical reagents on this system and so to investigate whether or not dihydrolipoamide dehydrogenase is associated with transport in trypanosomes. It would also be of interest to look for the presence and cellular location of the enzyme's normal substrate, lipoic acid, in *T. brucei*. To this end, we have developed a method using g.l.c.-mass spectrometry to detect lipoic acid as its oxidized lipoate methyl ester (Danson *et al.*, 1985).

In conclusion, there is growing evidence that dihydrolipoamide dehydrogenase has a cellular function in addition to its established role in the 2-oxo acid dehydrogenase complexes. It is considered that further studies of the enzyme in the archaeobacteria and *T. brucei*, where the multienzyme complexes are not found, will be valuable in identifying this new function.

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