Kinetic and physical properties of the L-malate–NAD⁺ oxidoreductase from *Methanospirillum hungatii* and comparison with the enzyme from other sources

A. C. STORER, G. D. SPROTT and W. G. MARTIN

Division of Biological Sciences, National Research Council of Canada, Ottawa, Canada K1A 0R6

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The L-malate–NAD⁺ oxidoreductase of *Methanospirillum hungatii* was purified to homogeneity by using Blue Sepharose and ADP–Sepharose affinity chromatography. The molecular weight was estimated as $61\,700 \pm 1900$ by gel filtration and $64\,200 \pm 1200$ by ultracentrifugation. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis indicated that the protein is composed of two polypeptide chains, each corresponding to $31\,350 \pm 2150$ daltons. Inhibition patterns obtained for malate, α -oxoglutarate and ADP established that the sequential reaction mechanism was ordered, with NADH serving as the first substrate. Intracellular concentrations of oxaloacetate approximated the K_m value of $27\,\mu\text{M}$, but NADH was present at less than K_m values. Comparison of the amino-acid composition of the L-malate–NAD⁺ oxidoreductase of *M. hungatii* and 22 others from prokaryotic and eukaryotic cells revealed a significant direct relationship between average hydrophobicity and the frequency of non-polar side chains, as well as a significant indirect relationship between average hydrophobicity and the polarity ratio. Calculations based on amino-acid-composition data indicated significant composition similarity between pairs of mammalian-cytoplasmic or pairs of mitochondrial L-malate–NAD⁺ oxidoreductases from various sources, but no significant composition similarity between any of the pairs of bacterial species examined.

Methanogenic bacteria are sufficiently different from typical bacteria to warrant a separate domain (Balch *et al.*, 1979). The proposal, made by Woese & Fox (1977), was to divide living organisms into three kingdoms; namely, the eubacteria, the eukaryotes, and the archaeobacteria, which would include the methanogens, extreme halophiles and the thermoacidophiles, *Sulfolobus* and *Thermoplasma*. Much of the evidence supporting this concept has come from sequence characterization of 16S ribosomal RNA (Fox *et al.*, 1977).

Recently we reported the partial purification and characterization of the L-malate–NAD⁺ oxidoreductase from *Methanospirillum hungatii* (Sprott *et al.*, 1979). The enzyme was different from the L-malate–NAD⁺ oxidoreductase examined by other workers in that, under physiological conditions, it functions as a unidirectional enzyme and it is inhibited *in vitro* by α -oxoglutarate.

In studies of evolutionary relationships and

bacterial taxonomy this enzyme may be of value because of its presence in most organisms, including both mitochondrial and cytoplasmic forms in eukaryotic cells. The present study was designed to compare the *M. hungatii* enzyme with those from other sources with respect to reaction mechanism and physical properties.

Materials and methods

Cell growth

Methanospirillum hungatii GPI was grown in 12-litre fermenter culture as described previously (Sprott *et al.*, 1979). Fresh reducing agent was added daily.

Enzyme purification

The L-malate–NAD⁺ oxidoreductase was partially purified by using $(\text{NH}_4)_2\text{SO}_4$ fractionation, gel filtration and Blue Sepharose CL-6B chromatography as described previously (Sprott *et al.*, 1979). The pooled active fraction from the Blue Sepharose

Abbreviation used: SDS, sodium dodecyl sulphate.

step was diluted 1:10 with water and applied again to the Blue Sepharose column. After washing with 25 ml of 0.01 M-Tris/HCl, pH 8.0, containing 1 mM-EDTA, the enzyme activity was eluted with 4 M-NaCl dissolved in the same solution to give a recovery of 141%. Salt was decreased to about 4 mM from the active fractions by using an ultrafiltration cell (Amicon PM 10 membrane), with full recovery of enzyme activity. The enzyme suspended in 0.02 M-Tris/HCl (pH 8.0)/1 mM-EDTA was bound to a column of 2',5'-ADP-Sepharose 4B (Pharmacia) of bed dimensions 1 cm $\times$ 10 cm with a binding efficiency of 70%. Enzyme activity was eluted with 0.2 mM-NADH in 0.02 M-Tris/HCl, pH 8.0.

Enzyme assay and storage

The purified enzyme was assayed as described previously (Sprott *et al.*, 1979) (at pH 8.0) and stored for several weeks at 4°C in 0.05 M-Tris/HCl, pH 8.0, containing 1 M-NaCl without appreciable loss of activity.

Ultracentrifuge analysis

The molecular weight was determined in a Beckman model E ultracentrifuge, by using a photoelectric scanner and multiplex accessory, by the conventional sedimentation-equilibrium method. A relatively short fluid column length (~ 3 mm) in double-sector centrifuge cells with sapphire windows was used. The procedure closely paralleled that described by Chervenka (1969). Runs were made at 20°C, in 0.5 M-NaCl, at 10000, 14000 and 17000 rev./min ($r_{av.} = 6.9$ cm) until unchanging patterns (scanned at 280 nm) were observed (~ 20 h). Baselines were established by running at 52000 rev./min and then reverting back to the equilibrium speed.

Electrophoresis

SDS/polyacrylamide-gel electrophoresis using 7% (w/v) gels was as described by Weber & Osborn (1969). The buffer chambers contained 0.1 M-phosphate buffer, pH 7.0, and 0.1% SDS. Purified enzyme or molecular-weight standards were dissolved in the same solution as used in the buffer chambers except for a 10-fold dilution of the buffer, and the inclusion of 40 mM-dithiothreitol and 20% (w/v) sucrose. After heating for 5 min in a boiling-water bath, samples were layered on the gels and electrophoresis begun (8 mA/gel).

Amino-acid analysis

Enzyme from the final purification step was washed with water, concentrated on an Amicon filter (PM 10), then hydrolysed in 6 M-HCl for 24 h or 72 h at 110°C in tubes sealed under vacuum. The acid was removed on a rotary evaporator at 37°C and the residue dried over KOH pellets *in vacuo*. Cystine plus cysteine were estimated as cysteic acid on

samples that had been oxidized at 0°C with performic acid (Hirs, 1967). Tryptophan was determined from the tyrosine/tryptophan ratio obtained by u.v. absorption (Edelhoch, 1967) and the known tyrosine content. The threonine and serine contents were corrected for losses sustained during hydrolysis.

Partial specific volume

The partial specific volume was calculated from the amino-acid composition as described by Cohn (1943).

Analysis of amino-acid-composition data

Data were analysed with an IBM 260/67 computer. The polarity ratio, the frequency of non-polar side chains, and the average hydrophobicity were calculated as described by Bigelow (1967). An estimate in sequence similarities for 23 L-malate-NAD⁺ oxidoreductases was made as described by Cornish-Bowden (1979).

Intracellular concentrations of NADH and oxaloacetate

M. hungatii GPI was grown in 100 ml portions of pre-reduced SA medium in 1-litre storage bottles modified as described by Balch & Wolfe (1978). Cells in late exponential phase were harvested anaerobically in 15 ml glass Corex tubes modified so a butyl-rubber stopper could be held in place with an aluminium seal. Tubes were centrifuged at 13000 g for 15 min at room temperature. Unwashed cell pellets (18 mg dry wt.) were extracted by modifying the method of Williamson & Corkey (1969). Oxaloacetate was extracted by injecting 2.0 ml of 6% (v/v) HClO₄. The sample was mixed, centrifuged in glass tubes at 9770 g for 10 min, and the pellet washed with 1.0 ml of 6% HClO₄. The combined supernatants were adjusted to pH 6.0 with 3.0 M-K₂CO₃ containing 0.5 M-triethanolamine base. KClO₄ was removed by centrifugation. NADH was extracted from anaerobic pellets by adding 2.0 ml of 0.33 M-KOH in 100% ethanol. The sample was mixed, heated for 1 min at 37°C, neutralized to pH 8.5, and centrifuged at 9770 g for 10 min. The pellet was washed with 1.0 ml of 50 mM-triethanolamine buffer, pH 8.2. The respective combined supernatants (3.0 ml) were assayed for oxaloacetate with malate dehydrogenase (Sigma) and for NADH with lactate dehydrogenase (Worthington) as described by Williamson & Corkey (1969). Measurements were made with a Perkin-Elmer recording spectrophotometer set for 0.1 or 0.05 A units for full-scale deflection. Assays were conducted in 1.0 ml volumes with triethanolamine buffer, pH 7.4. Standard curves were constructed by assaying known amounts of the substrate to be tested in the presence and absence of cell extract. Measurements were

based on dry weights obtained with cell pellets washed with water. Weights were determined after 24 h at 62°C, and corrected for ash by heating at 450°C for 8 h.

Results

Enzyme purity

L-Malate-NAD⁺ oxidoreductase was isolated from the 150 000 g supernatant obtained after breakage of the cells in a French pressure cell [82.8–110.4 MPa (12 000–16 000 lb/in²)]. The initial steps in the purification, namely (NH₄)₂SO₄ fractionation, gel filtration, Blue Sepharose column chromatography and were performed with similar recoveries and specific activities to those reported previously (Sprott *et al.*, 1979). The enzyme preparation, after the first passage through Blue Sepharose, appeared as a yellow-coloured extract. The pigment was removed by a second Blue Sepharose step, yielding a product that on SDS/polyacrylamide-gel electrophoresis gave several protein bands. Because the L-malate-NAD⁺ oxidoreductase of *M. hungatii* is known to show competitive inhibition by ADP with respect to NADH (Sprott *et al.*, 1979), an ADP-Sepharose affinity step was included in the purification. The enzyme bound to ADP-Sepharose and was eluted with 0.2 mM-NADH. This final preparation yielded a single protein band, corresponding to $31\,350 \pm 2150$ daltons (two separate experiments) on SDS/polyacrylamide-gel electrophoresis (Fig. 1). The purified enzyme exhibited a specific activity of 62 μ mol of NADH oxidized/min per mg, with an increase in specific activity of 14 000-fold compared with the crude extract. In view of the degree of purification required, the overall yield of 21% seems high. This is caused in part by recoveries in activity often up to 140% after each of the Blue Sepharose steps.

Further evidence for protein homogeneity was obtained by ultracentrifugation (Fig. 2). During the course of the runs, no evidence of low-molecular-weight components was observed, but at high r^2 values (where r is the distance from the centre of rotation), a break in the curves occurred, suggesting some aggregation of the enzyme.

The molecular weight was estimated from equilibrium runs at three rotor speeds (Fig. 2). From statistical analysis of the data the mol.wt. was $64\,200 \pm 1200$, using the value for partial specific volume of 0.74 cm³/g computed from the amino-acid composition. This molecular weight agrees, within the error of measurement, with the value of $61\,700 \pm 1900$ obtained previously (Sprott *et al.*, 1979) by gel filtration and confirmed with the present enzyme preparation. Since the molecular weight obtained from SDS/polyacrylamide-gel electrophoresis was 31 350, it would appear that the

Fig. 1. SDS/polyacrylamide-gel electrophoresis of purified *M. hungatii* L-malate-NAD⁺ oxidoreductase. Protein (54 μ g), applied to a 7% (w/v) polyacrylamide gel, was run towards the anode, Bromophenol Blue being used to mark the front. Molecular-weight standards were: bovine albumin monomer (mol.wt. 68 000) and dimer (136 000), ovalbumin monomer (43 000) and dimer (86 000), carbonic anhydrase (30 000) and human haemoglobin (16 000).

Fig. 2. Sedimentation-equilibrium plots of purified *M. hungatii* L-malate-NAD⁺ oxidoreductase. Data were collected by centrifuging the sample at three speeds (O, 10 000 rev./min; □, 14 000 rev./min; △, 17 000 rev./min) at 20°C and scanning at 280 nm.

L-malate–NAD⁺ oxidoreductase is composed of two equally sized subunits.

Kinetics

The L-malate–NAD⁺ oxidoreductase isolated from *M. hungatii* follows a sequential kinetic mechanism (Sprott *et al.*, 1979). In order to determine whether or not the addition of substrates is random or ordered, and if ordered what the order of addition is, product- and substrate-analogue-inhibition studies were carried out. The results of these studies are given in Table 1; some of these data were reported previously (Sprott *et al.*, 1979). The reaction was studied in the direction of oxaloacetate reduction, since the reverse reaction, malate oxidation, was too slow to be detected, even at high concentrations of both L-malate (200 mM) and NAD⁺ (2 mM). NAD⁺ at concentrations up to 5 mM produced no significant inhibition of the reduction of oxaloacetate, whereas L-malate was an effective inhibitor of this reaction, giving 50% inhibition at 5 mM-malate. The substrate analogues used were 2-oxoglutarate for oxaloacetate and ADP for NADH. Both inhibited the enzyme-catalysed reaction.

The inhibition data were analysed as described by Cornish-Bowden (1974). The constants in Table 1 refer to the general kinetic equation:

$$v = \frac{V_{\max} \cdot s}{K_M \left(1 + \frac{i}{K_I}\right) + s \left(1 + \frac{i}{K'_I}\right)}$$

where K_M refers to the apparent Michaelis constant of the enzyme for the varied substrate (S) at a fixed

concentration of the second substrate, s and i are the varied substrate and inhibitor concentrations respectively, and K_I is the competitive and K'_I the uncompetitive inhibition constants for the inhibitor I.

Typically, supernatant L-malate–NAD⁺ oxidoreductases appear to be inhibited less by high oxaloacetate concentrations than are the corresponding mitochondrial isoenzymes (Kitto & Lewis, 1967). In this respect the *M. hungatii* enzyme resembles the supernatant enzymes of eukaryotic cells, since no significant change in reaction velocity occurred between 0.3- and 1.0 mM-oxaloacetate (results not shown). The inhibition caused by excess NADH was found to be less dramatic than previously reported (Sprott *et al.*, 1979). An increase in NADH concentration from the optimum of 0.3 to 0.6 mM resulted in only a 25% decrease in reaction velocity.

Intracellular NADH and oxaloacetate

Methanogen extracts contain several fluorescent cofactors that are not found in other bacteria or tissues (Balch *et al.*, 1979). These cofactors resulted in a high background fluorescence (excitation at 340 nm, emission at 465 nm), severely restricting the amount of extract that could be added to the assay. Consequently, cells were concentrated by centrifuging anaerobically before extraction, and NADH or oxaloacetate quantified by measuring ΔA_{340} . Oxaloacetate was present at 62 nmol/g cell dry wt. Assuming an internal space of 3 μ l/mg, this would correspond to an internal concentration of 20 μ M, close to the apparent K_m of 27 μ M. NADH concentrations were below the detection level of these methods, and it can therefore be estimated that they

Table 1. Characterization of the L-malate–NAD⁺ oxidoreductase from *M. hungatii* with respect to inhibition type and inhibition constants for several inhibiting compounds

Abbreviations used: OAA, oxaloacetate; comp., competitive; uncomp., uncompetitive.

Inhibitor	Varied substrate	Concn. of second substrate (mM)	Type of inhibition observed	Inhibition patterns predicted by various kinetic models				Random
				Inhibition constants*		Ordered with first substrate		
				K_I (mM)	K'_I (mM)	NADH	OAA	
Malate	OAA	0.2	Mixed	7.5	16	Mixed	Mixed	Comp.
	NADH	0.2	Mixed	≥ 30	6	Mixed	Mixed	Mixed
NAD ⁺⁺	OAA	0.2	—	—	—	Mixed	Mixed	Mixed
	NADH	0.2	—	—	—	Comp.	Comp.	Comp.
α -Oxoglutarate	OAA	0.2	Mixed	0.18	0.55	Mixed	Uncomp.	Comp.
	NADH	0.2	Uncomp.	—	0.5	Uncomp.	Mixed	Mixed
ADP	OAA	0.2	Mixed	1.25	7	Mixed	Comp.	Mixed
	NADH	0.2	Comp.	0.5	—	Comp.	Mixed	Comp.

* K_I is the competitive and K'_I the uncompetitive inhibition constant (see Cornish-Bowden, 1974).

† Inhibition of the enzyme by NAD⁺ was undetectable.

Table 2. Amino-acid composition (residues/mol) of L-malate–NAD⁺ oxidoreductase

Sources of data are as follows (the soluble isoenzyme is denoted by s-, the mitochondrial by m-): *Methanospirillum hungatii*, the present study; *Thermus flavus*, Saiki *et al.* (1978); *Escherichia coli*, Murphey *et al.* (1967b); extreme halophile, Mevarech *et al.* (1977); *Thermus aquaticus*, Biffen & Williams (1976); *Neurospora crassa* m-, Munkres & Richards (1965); *Bacillus subtilis* (residues/0.5 mol), Yoshida (1965); *Pseudomonas testosteroni*, You & Kaplan (1975); *Mycobacterium* sp., Imai (1978); chicken heart s-, Kitto & Kaplan (1966); *Mycobacterium phlei*, Tyagi *et al.* (1977); *Saccharomyces cerevisiae* s-, Hägele *et al.* (1978); *S. cerevisiae* m-, Hägele *et al.* (1978); chicken heart s-, Kitto & Kaplan (1966); chicken heart m-, Kitto & Kaplan, (1966); ox heart s-, Siegel & England (1962); ox heart m-, Siegel & England (1962); pig heart m-, Noyes *et al.* (1974); pig heart s-, Wade (1971); horse heart m-, Thorne (1962); tuna heart m-, Kitto & Lewis (1967); salmon heart AA s- and salmon muscle BB s-, Bailey *et al.* (1970); rat liver m-, Kuan *et al.* (1979).

Amino acid	<i>M. hungatii</i>	<i>T. flavus</i>	<i>E. coli</i>	Halophile	<i>T. aquaticus</i>	<i>N. crassa</i> m-	<i>B. subtilis</i>	<i>P. testosteroni</i>	<i>Mycobacterium</i> sp.	<i>M. phlei</i>	<i>S. cerevisiae</i> s-	<i>S. cerevisiae</i> m	Chicken s-	Chicken m-	Ox s-	Ox m-	Pig m-	Pig s-	Horse m-	Tuna m-	Salmon AA s-	Salmon BB s-	Rat liver m-
Asp	52.8	50.1	44.5	113.8	58.0	35.3	62.2	67.7	38.2	73.0	68.3	53.3	68	52	47.5	43.8	50	78	42	60	78	70	52
Thr	33.0	24.0	32.6	40.1	27.9	21.2	53.8	30.6	24.9	22.2	22.3	28.7	30	44	16.7	34.7	42	32	42	40	20	28	44
Ser	40.6	14.7	30.0	44.7	30.7	21.1	46.0	29.5	80.7	21.3	51.1	32.8	39	42	20.8	25.9	36	44	36	40	50	48	28
Glu	55.9	60.9	63.5	104.8	82.2	29.8	76.8	58.0	69.9	70.7	49.7	46.9	60	60	38.3	50.3	50	54	50	50	52	54	52
Pro	20.5	28.7	26.0	34.7	32.7	17.3	30.0	30.7	15.2	19.6	35.8	33.3	29	41	14.6	36.7	46	24	44	42	24	28	42
Gly	63.3	49.2	62.5	85.9	68.6	35.6	71.2	64.6	76.7	38.8	45.7	49.3	59	61	29.1	45.3	58	56	54	58	48	46	56
Ala	56.7	88.8	61.0	81.3	89.0	34.4	59.0	107.2	44.5	67.4	40.9	48.6	60	66	38.1	50.9	66	64	66	72	72	84	64
Val	40.9	45.2	56.5	71.0	58.2	26.3	65.0	37.7	25.9	51.6	52.0	45.1	53	48	32.8	42.1	54	52	54	64	56	58	56
Met	13.3	20.3	7.3	10.9	2.2	3.3	16.8	21.4	0.0	49.8	12.5	6.7	14	13	10.1	8.2	12	16	14	12	20	12	12
Ile	42.4	27.5	31.0	37.8	36.8	15.6	43.6	33.0	13.0	42.0	49.4	39.5	42	40	24.5	31.5	42	38	36	32	38	36	46
Leu	41.0	55.9	66.5	53.1	68.8	28.2	61.6	63.3	24.3	46.0	61.0	52.3	59	57	39.1	44.4	56	64	60	60	64	56	58
Tyr	13.4	12.1	10.1	20.4	0.0	8.2	30.6	14.2	6.9	14.3	14.9	6.3	16	9	8.5	7.1	10	16	10	8	14	8	10
Phe	18.2	18.0	22.1	21.2	24.9	12.0	16.6	23.1	11.9	30.6	15.8	20.7	24	30	13.6	23.3	22	22	22	20	20	22	24
His	11.4	6.8	5.2	19.1	12.7	6.6	4.2	9.8	32.7	36.7	16.5	14.4	11	11	4.7	7.9	10	8	12	12	16	14	10
Lys	29.4	32.3	37.2	21.2	35.3	28.0	42.0	36.6	8.3	67.3	30.1	45.1	57	49	46.5	34.1	52	62	52	40	48	48	54
Arg	27.7	35.1	16.6	38.0	44.6	10.9	30.0	16.9	21.1	14.7	23.6	17.7	20	20	13.0	12.1	16	20	16	18	28	16	16
Trp	6.8	13.0	3.5	6.3	3.0	1.1	0.0	10.2	7.0	4.4	0.0	0.0	12	0	7.9	0.94	0	10	0	0	8	0	0
Cys	5.5	4.0	6.0	0.0	6.8	4.3	0.9	3.5	2.7	6.9	8.0	1.9	8	14	6.1	12.2	14	10	14	14	12	16	8
X*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.7	0.0	0.0	0.0	0	0	0.0	0.0	0	0	0	0	0	0	0
Sum	574.8	586.6	582.1	804.3	682.4	339.2	714.3	658.0	511.6	677.3	597.6	547.6	661.0	657.0	411.9	511.4	636	660	624	642	668	662	632
Molecular weight	61 700	72 000	60 000	84 000	73 500	54 000	148 000	70 000	86 860	86 860	75 000	68 000	67 000	67 000	52 000	62 000	67 000	—	70 000	68 000	68 000	68 000	70 000

* Unidentified amino acid residue.

Table 3. *Parameters calculated from amino-acid-composition data for the L-malate-NAD⁺ oxidoreductases from various species*

Parameters were calculated according to Bigelow (1967) with the aid of a computer, and listed in order of increasing hydrophobicity. For full generic names of organisms, see Table 2.

No.	Organism	Residues (acidic/basic)	Polarity ratio	Non-polar- side-chain frequency	Average hydrophobicity (kcal*/residue)
1	<i>Mycobacterium</i> sp.	3.68	1.473	0.207	0.651
2	Halophile	3.69	1.097	0.304	0.916
3	<i>T. aquaticus</i>	1.76	0.833	0.329	1.038
4	<i>N. crassa</i> m-	1.67	1.007	0.320	1.049
5	<i>M. hungatii</i>	1.90	0.933	0.322	1.051
6	Salmon BB s-	1.63	0.867	0.323	1.075
7	<i>B. subtilis</i>	1.99	1.046	0.346	1.082
8	<i>P. testosteroni</i>	2.35	0.729	0.322	1.092
9	Salmon AA s-	1.71	0.878	0.335	1.099
10	Tuna m-	1.90	0.765	0.352	1.102
11	<i>M. phlei</i>	1.75	0.940	0.308	1.111
12	Chicken heart m-	1.62	0.828	0.342	1.122
13	<i>E. coli</i>	2.01	0.753	0.371	1.128
14	<i>S. cerevisiae</i> m-	1.68	0.887	0.360	1.130
15	Pig heart s-	1.61	0.931	0.342	1.139
16	<i>T. flavus</i>	1.65	0.754	0.342	1.143
17	<i>S. cerevisiae</i> s-	2.20	0.866	0.383	1.148
18	Ox heart m-	2.04	0.773	0.364	1.150
19	Chicken heart s-	1.66	0.879	0.356	1.152
20	Ox heart s-	1.44	0.949	0.342	1.162
21	Pig heart m-	1.41	0.777	0.362	1.171
22	Horse heart m-	1.35	0.773	0.362	1.176
23	Rat liver m-	1.49	0.782	0.373	1.196

* To convert to SI units, 1 kcal $\equiv$ 4.18 kJ.

Fig. 3. *Relation between average hydrophobicity, the frequency of non-polar side chains (NPS) and the polarity ratio (ρ)*
Each datum point is given with a number that corresponds to that given alongside the organisms reported in Table 3. (To convert to SI units, 1 kcal $\equiv$ 4.18 kJ.)

must have been at least an order of magnitude less than the K_m value of 80 μ M.

Amino-acid composition

In Table 2 the amino-acid composition of the L-malate-NAD⁺ oxidoreductase of *M. hungatii* is presented with data from other sources, recalculated, when necessary, as residues/mol. The data for *M. hungatii* were obtained in separate samples of the purified enzyme hydrolysed in duplicate for 24 and 72 h. The number of residues/mol varied less than 5% for each amino acid between duplicate samples. As shown by Murphey *et al.* (1967a), the L-malate-NAD⁺ oxidoreductases can be separated on the basis of molecular weight into two categories, namely, those of about 60 000 and those of about 117 000. The latter category is present in the Gram-positive bacteria of the order Eubacteriales. Data for the *Bacillus subtilis* enzyme of the high-molecular-weight form is shown as residues/0.5 mol of enzyme from the calculations reported here. The enzyme of *Neurospora crassa* isolated by Munkres & Richards (1965) appears to have been of mitochondrial origin, although a cytoplasmic isoenzyme also exists (Kitto *et al.*, 1967).

The amino-acid-composition data for each L-malate-NAD⁺ oxidoreductase was used to calculate several structural parameters by the methods given by Bigelow (1967). Fisher's polarity ratio (ρ) is the ratio of the volume occupied by polar residues to that occupied by the non-polar residues; Waugh's non-polarity index, NPS, is the frequency of non-polar side chains; average hydrophobicity is the total hydrophobicity divided by the number of residues; and the ratio of acidic/basic residues is given as (glutamic acid + aspartic acid)/(lysine + arginine). The L-malate-NAD⁺ oxidoreductases are listed in Table 3 according to increasing values of average hydrophobicity. The enzymes appear to vary in these parameters much more than do other related proteins such as haemoglobin, cytochrome *c* or haemocyanins (Bigelow, 1967).

Bigelow (1967) has shown that average hydrophobicity and NPS are directly related for a wide variety of proteins, but he was unable to show an indirect relationship for average hydrophobicity and ρ . Linear regression analysis gave a coefficient of determination, r^2 , of 0.84 between average hydrophobicity and NPS for the enzyme data shown in Fig. 3(a), and 0.75 for the relationship between

Table 4. Values of $\sqrt{S\Delta n}$ for L-malate-NAD⁺ oxidoreductases

Amino-acid-composition data (Table 2) were analysed with a computer to calculate $S\Delta n$ values as described by Cornish-Bowden (1979). Full generic names of organisms are given in Table 2.

		$\sqrt{S\Delta n}$																						
Organism	Organism ...	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	<i>M. hungatii</i>	0																						
2	<i>T. flavus</i>	37	0																					
3	<i>E. coli</i>	29	33	0																				
4	Halophile	52	58	55	0																			
5	<i>T. aquaticus</i>	40	30	30	48	0																		
6	<i>N. crassa</i> m-	20	38	31	61	47	0																	
7	<i>B. subtilis</i>	28	45	27	48	43	33	0																
8	<i>P. testosteronei</i>	42	27	39	58	38	44	50	0															
9	<i>Mycobacterium</i> sp.	50	73	63	68	68	57	61	73	0														
10	<i>M. phlei</i>	50	49	53	72	62	40	58	54	82	0													
11	<i>S. cerevisiae</i> s-	31	51	38	59	51	30	37	55	62	54	0												
12	<i>S. cerevisiae</i> m-	25	40	27	58	42	21	32	44	64	43	23	0											
13	Chicken s-	25	38	24	56	41	17	31	40	64	41	29	14†	0										
14	Chicken m-	26	38	21	61	38	17	32	40	61	50	35	18	23	0									
15	Ox s-	31	40	35	66	50	19	39	47	68	33	33	23	14†	28	0								
16	Ox m-	26	37	25	61	42	17	34	43	62	47	33	19	23	12†	26	0							
17	Pig s-	35	42	33	61	48	26	38	42	71	42	33	23	15†	31	17	32	0						
18	Pig m-	31	39	25	66	43	21	35	42	68	51	36	19	24	12†	29	15†	31	0					
19	Horse m-	34	38	24	70	43	22	37	43	68	51	38	22	27	14†	29	17	33	9†	0				
20	Tuna m-	32	36	23	57	37	26	34	37	65	53	34	23	26	19	33	21	30	17	18	0			
21	Salmon AAs-	33	38	35	57	42	27	41	39	66	43	28	26	22	34	24	35	18	35	37	29	0		
22	Salmon BB s-	33	33	33	57	36	28	41	34	66	46	36	27	25	29	29	31	23	30	31	23	16†	0	
23	Rat liver m-	32	39	24	65	42	25	34	42	72	48	37	18	23	16†	29	19	30	9†	13†	20	36	32	0

† Significant composition similarity according to 95% confidence test of Cornish-Bowden (1979), i.e. $S\Delta n$ less than 42% of the length of the shorter sequence.

average hydrophobicity and ρ (Fig. 3b). Analysis omitting the two extreme cases (*Mycobacterium* sp. and the extreme halophile) still gave an r^2 value of 0.51 (significant at $P < 0.01$) when average hydrophobicity was a function of NPS, but now the r^2 value was only 0.16 (not significant, $P \sim 0.08$) when average hydrophobicity was considered as a function of ρ . There is, however, no obvious reason to exclude these two cases.

A different method to compare amino-acid-composition data, by estimation of sequence similarities, has been described by Cornish-Bowden (1979). The indexes of composition difference, $S\Delta n$, were calculated from the data in Table 2 by using a correction for differences in length of the polypeptide chains (Cornish-Bowden, 1979). Significant composition similarities (95% confidence) were found between pairs of the L-malate-NAD⁺ oxidoreductases from several eukaryotic sources (Table 4). For the mitochondrial enzymes these were ox and chicken, pig and chicken, horse and chicken, rat and chicken, pig and ox, horse and pig, rat and pig, and rat and horse. For the cytoplasmic enzymes, significant similarities were found for ox and chicken, pig and chicken and between the salmon enzymes. In addition, chicken cytoplasmic and yeast mitochondrial L-malate-NAD⁺ oxidoreductases showed significant similarity. No significant similarities were found within the bacterial sources.

Discussion

The inhibition results for the *M. hungatii* L-malate-NAD⁺ oxidoreductase (Table 1) are consistent with a compulsory ordered mechanism in which NADH is the first substrate to bind (Cleland, 1963a,b,c; Heyde & Ainsworth, 1968). This mechanism is identical with that reported for the enzyme from a wide variety of sources (Silverstein & Sulebele, 1969a,b; Heyde & Ainsworth, 1968; Raval & Wolfe, 1962a,b).

An unusual property of the enzyme isolated from *M. hungatii* is its K_{IA} for NADH of 21 mM (Sprott *et al.*, 1979). For a simple ordered mechanism, K_{IA} is the dissociation constant of the enzyme-NADH complex. Published values of K_{IA} obtained from initial-rate measurements for a variety of L-malate-NAD⁺ oxidoreductases and dehydrogenases in general are consistently in the range 10^{-7} – 10^{-5} M (Dalziel, 1975). The dissociation constants that have been measured for dehydrogenase-enzyme-NAD⁺ complexes are usually one to two orders of magnitude larger than the corresponding constants for the enzyme-NADH complexes. Thus the inability to detect the oxidation of malate by the enzyme from *M. hungatii* is possibly due to a high dissociation constant for the enzyme-NAD⁺ complex. By the above argument the dissociation constant is predicted to be very large and in the range 0.2–2 M. This

prediction is supported by the absence of inhibition of the enzyme by NAD⁺ at concentrations of up to 5 mM. A high dissociation constant would ensure that, under physiological conditions, the reaction would be effectively unidirectional, i.e. the rate of oxidation of malate catalysed by the enzyme is insignificant when compared with the catalysed rate of reduction of oxaloacetate. The apparent irreversibility of this catalytic step probably plays an important role in the overall metabolism of the organism. Thus *M. hungatii* has been able to fulfil its requirement of irreversibility by the evolution of a unique L-malate-NAD⁺ oxidoreductase, i.e. an enzyme with an unusually high dissociation constant for NAD⁺. This irreversibility is achieved at the expense of catalytic efficiency, since the dissociation constant for NADH is also very high. Under normal physiological conditions the enzyme is far from saturated with NADH; however, this is overcome to a certain extent by the low K_m for oxaloacetate (27 μ M). The high affinity of the enzyme-NADH complex for oxaloacetate ensures that the bound reduced coenzyme is trapped in the enzyme by the binding of oxaloacetate. The concept of unidirectional or one-way enzymes has been discussed by Jencks (1975).

In spite of the similarity of the reaction mechanism of L-malate-NAD⁺ oxidoreductases from all sources, as discussed above, the various organisms have evolved enzymes that fulfil their own particular needs. For example, the enzyme of an unidentified extreme halophile is inactivated at low ionic strengths (Mevarech *et al.*, 1977), that of *Mycobacterium* sp. is particulate, requiring FAD⁺ and phospholipid for maximum activity (Imai, 1978), and the enzyme of *Thermus aquaticus* is relatively thermostable, exhibiting an optimum temperature for activity of 70°C (Biffen & Williams, 1976).

On the basis of physical properties the methanogen enzyme is not unusual. It belongs to the low-molecular-weight category, as described by Murphey *et al.* (1979a), and is composed of two similarly sized subunits. Further, the partial specific volume was in the usual range, namely 0.74 cm³/gm.

Comparisons in amino-acid composition have been made previously among several L-malate-NAD⁺ oxidoreductases, although generally little attempt has been made to define these differences mathematically. Exceptions are the work of Biffen & Williams (1976) and Saiki *et al.* (1978), when they compared data from several sources according to acidic and basic residues, non-polar side chains, polarity ratio and average hydrophobicity. They concluded that no striking differences in the above parameters occurred for the data analysed. A larger sampling of data, arranged according to change in the average hydrophobicity (Table 3), indicates a

possible trend to increasing hydrophobicity from the bacterial to higher forms. Further, the direct relationship found between average hydrophobicity and non-polar side chains supports the conclusion of Bigelow (1967) that these parameters are measures of the same property. The limited value of determining average hydrophobicity as a measure of change among species is indicated by the inability to group the mitochondrial and soluble (cytoplasmic) enzymes into two separate clusters.

On examination of the calculated values of Δn , two groups of L-malate-NAD⁺ oxidoreductases are found with significant composition similarities (>95% confidence). One group consists of the mitochondrial enzymes from horse, pig, rat, ox and chicken, whereas the other group contains the soluble enzymes from pig, ox, salmon and chicken (the amino-acid composition of the horse and rat soluble enzymes are not available for comparison). The tuna mitochondrial enzyme was also found to be very similar to the mitochondrial group. The two soluble salmon enzymes, known to be related in amino-acid sequence (Bailey *et al.*, 1970), possess a significant composition similarity (>95%), as do several other pairs of mitochondrial or soluble L-malate-NAD⁺ oxidoreductases (see the Results section). These results support the conclusion that compositional similarities occur among the mitochondrial, as well as among the supernatant enzymes of various species (Kaplan & Ciotti, 1961; reviewed by Banaszak & Bradshaw, 1975). A further significant compositional similarity of >95% is found between the chicken heart soluble enzyme and the *Saccharomyces cerevisiae* mitochondrial enzyme. However, the significance of this comparison is in question because these two proteins differ in size by approx. 100 residues, and, as Cornish-Bowden (1979) points out, size differences of greater than 20 residues make Δn an unsatisfactory index, i.e. for these two proteins the size correction applied to Δn is almost twice the final value of Δn . No significant similarities in composition were found between the L-malate-NAD⁺ oxidoreductase of *M. hungatii* and the enzymes from other sources, or between any of the pairs of enzymes of prokaryotic origin. The latter observation is consistent with the conclusion reached by Kaplan & Ciotti (1961) that the enzymes from these sources have diverged considerably.

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