

A soluble NADH dehydrogenase (NADH: ferricyanide oxidoreductase) from *Thermus aquaticus* strain T351

Kevan A. J. WALSH, R. M. DANIEL* and H. W. MORGAN
School of Science, University of Waikato, Hamilton, New Zealand

(Received 27 July 1982/Accepted 28 October 1982)

A soluble NADH dehydrogenase (NADH:ferricyanide oxidoreductase) has been obtained by simple disruption of cells of *Thermus aquaticus* strain T351, and purified. The enzyme is of low molecular mass, 50 000 Da, and displays many of the properties of the membrane-bound enzyme, including inhibition by both NADH and ferricyanide, and the same K_m for ferricyanide. The enzyme contains 0.05 mol of FMN, 0.16 mol of labile sulphur and 2.2 mol of iron per mol of protein. The enzyme is inhibited by NAD and cupferron competitively with ferricyanide, and by ATP (but not ADP) competitively with NADH. The enzyme is particularly thermostable, having a half-life at 95°C of 35 min. The effect of temperature on the molar absorption coefficient and the stability of NADH was determined.

Studies of isolated, individual, electron-transport system components often involve solubilization techniques which result in altered catalytic and physical properties. The discovery of an apparently soluble NADH dehydrogenase (NADH:ferricyanide oxidoreductase, EC 1.6.99.3) (Hickey & Daniel, 1979) from an extremely thermophilic bacterium seemed therefore to offer a particularly good opportunity to investigate an electron transport system component which would not require harsh pretreatment for study, and which would be intrinsically stable.

Although the thermostability of NADH oxidase of *Bacillus stearothermophilus* was thought mainly to reflect some property of the membrane itself (Wisdom & Welker, 1973), it is in fact clear that proteins from thermophiles are generally more stable than their counterparts from mesophiles (e.g. Koffler & Gale, 1957; Amelunxen & Lins, 1968), and it has been shown that the solubilized NADH dehydrogenase from *B. stearothermophilus* does indeed possess remarkable thermostability (Mains *et al.*, 1980). An NADH dehydrogenase from *Bacillus caldotenax* YT-G has also been solubilized and purified, and the effects of membrane lipids on the enzyme have been examined (Kawada *et al.*, 1981). A brief survey of respiratory proteins from two extreme thermophiles, *Thermus thermophilus* HB8 and *Bacillus caldolyticus*, showed that the K_m values of the Triton X-100-solubilized NADH dehydrogenases were very similar to those of membrane-

bound enzymes (Fee *et al.*, 1978). An NADH dehydrogenase activity has been reported for the thermophilic bacterium PS3, but this was used only as a measure of contamination in the purification of an alanine carrier (Hirata *et al.*, 1976, 1977). So although thermophilic organisms are a potential source of a stable preparation of NADH dehydrogenase, all preparations so far examined have required solubilization.

Experimental

Growth of bacteria and preparation of subcellular fractions

Thermus aquaticus strain T-351 was maintained, grown and harvested at 75°C, and cells were disrupted using a French pressure cell, and subcellular fractions prepared by differential centrifugation as described by Hickey & Daniel (1979).

Enzyme assay

NADH dehydrogenase activity was assayed with a Unicam SP.1800 spectrophotometer with the cell compartment maintained at 75°C. Cuvettes contained 2 μ mol of potassium ferricyanide, 9 μ mol of NADH, 0.1 ml of enzyme sample, and 17 mM-Tris/HCl buffer, pH 7.0, in a total volume of 3.0 ml. The non-enzymic rate was recorded, then the enzyme solution was added to the cuvette and rapidly mixed and the reaction rate recorded for 10–30 s; the reaction rate was linear over this period. The enzymic rate was determined by subtracting the non-enzymic rate from the linear rate obtained after

Abbreviation used: SDS, sodium dodecyl sulphate.

* To whom reprint requests should be addressed.

enzyme addition. Any inhibitors were present before enzyme addition. Controls were performed in an identical manner, except that autoclaved enzyme was used. A_{420} for potassium ferricyanide at 75°C and pH 7.0 was taken as $0.91 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (see the Results section). One unit of enzyme activity is defined as the amount of enzyme that results in the reduction of 1 μmol of ferricyanide/min.

Temperature effects on NADH and ferricyanide

NADH degradation was observed by following the absorbance decrease at 340nm. Stopped cuvettes contained 0.2mM-NADH in either 0.005M-Mes (4-morpholine-ethanesulphonic acid)/NaOH buffer or $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer at various pH values but with the ionic strength adjusted to 0.01M with NaCl. The cuvette was heated in the thermostated cell compartment of the Unicam SP.1800 spectrophotometer. The temperature of the solution in the cuvette was measured with a copper-constantan thermocouple. The decrease in pK_a with increase in temperature for Mes buffer is 0.011°C (Good *et al.*, 1966). The effect of temperature on the pK_a of $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer is negligible (Bates, 1962).

The effect of temperature on the molar absorption coefficient of NADH at 340nm was determined by incubating a stopped cuvette containing $6.0 \times 10^{-5}\text{M}$ -NADH in 17mM-Tris/HCl buffer, pH 9.0, at various temperatures. (At pH 9.0, degradation of NADH is negligible.) The effect on the molar absorption coefficient of $\text{K}_3\text{Fe}(\text{CN})_6$ at 420nm was similarly determined. The cuvette contained $8.05 \times 10^{-4}\text{M}$ - $\text{K}_3\text{Fe}(\text{CN})_6$ in 17mM-Tris/HCl buffer, pH 7.0.

Enzyme purification

A QAE-Sephadex A-50 column (2.6cm \times 30cm) was equilibrated at room temperature with 0.1M-NaCl in 50mM-Tris/HCl buffer, pH 8.0. The conductivity of the high-speed supernatant was adjusted to equal that of the column buffer. The solution was applied to the column, and the column was then washed with equilibrating buffer and sequentially with solutions of 0.15, 0.2 and 0.3M-NaCl in 50mM-Tris/HCl buffer, pH 8.0.

The 0.2M-NaCl fraction from the ion-exchange chromatography was concentrated by freeze-drying to a final volume of 1.5ml, and applied to a Sephadex G-150 column (1.6cm \times 70cm) equilibrated at 40°C with 5mM-Tris/HCl buffer, pH 7.0. The column was calibrated with molecular weight standards.

The active fractions from the gel-filtration chromatography were pooled, desalted, and applied to a hydroxyapatite column (2.6cm \times 30cm) equilibrated with distilled water at room temperature. The column was washed with distilled water, and then a

linear $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer gradient from 0 to 0.4M, pH 7.0, was applied. Fractions (5ml) were collected. The active fractions were pooled, desalted, and stored in liquid N_2 .

Isoelectric focusing

Isoelectric focusing was carried out on LKB Ampholine PAG plates, pH 3.5–9.5. Purified enzyme (7.4 μg) was focused on a Pharmacia Flatbed electrophoresis unit. The pH gradient was determined with a Pye surface electrode.

Enzyme activity was visualized by placing the PAG plate in a petri dish containing 1mM-*p*-iodonitrotetrazolium violet (Sigma) in 50mM-Tris/HCl buffer (pH 7.0)/ethanol (1:1, v/v). NADH was added to a concentration of 0.3mM, then the solution was incubated at 75°C for 10–20min. Enzyme activity appeared as a red band.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide gels were prepared and run by the method of Weber & Osborn (1969). Purified enzyme (90 μg) was electrophoresed at a current of 7mA per gel tube for 7h. Protein staining and destaining were carried out by the method of Weber & Osborn (1969).

Analytical methods

FMN and FAD were determined fluorometrically using the method of Burch (1957).

The non-haem iron content was determined by the method of Brumby & Massey (1967).

The acid-labile sulphide content was determined by the method of King & Morris (1967) adapted from the method of Fogo & Popowsky (1949).

Protein content of solutions containing subcellular particles was determined by a modification of the biuret method (Gornall *et al.*, 1949). Protein content of purified solutions was determined by the Coomassie Blue assay of Bradford (1976). Bovine serum albumin was used as a standard.

Electron spin resonance spectroscopy

E.s.r. spectra were recorded on a Varian E-line Century series spectrometer. Samples were reduced with either 0.3mM-NADH or 0.6mM-sodium dithionite. Samples were quickly cooled in a liquid N_2 insert Dewar (77K). Spectra were recorded at 9.08GHz with a field modulation frequency of 1000kHz.

Results

Effect of temperature on NADH

Under the conditions of the enzyme assay, the molar absorption coefficient of NADH at 340nm decreased linearly between 20 and 75°C by 0.13% for each 1°C rise in temperature and at 75°C was

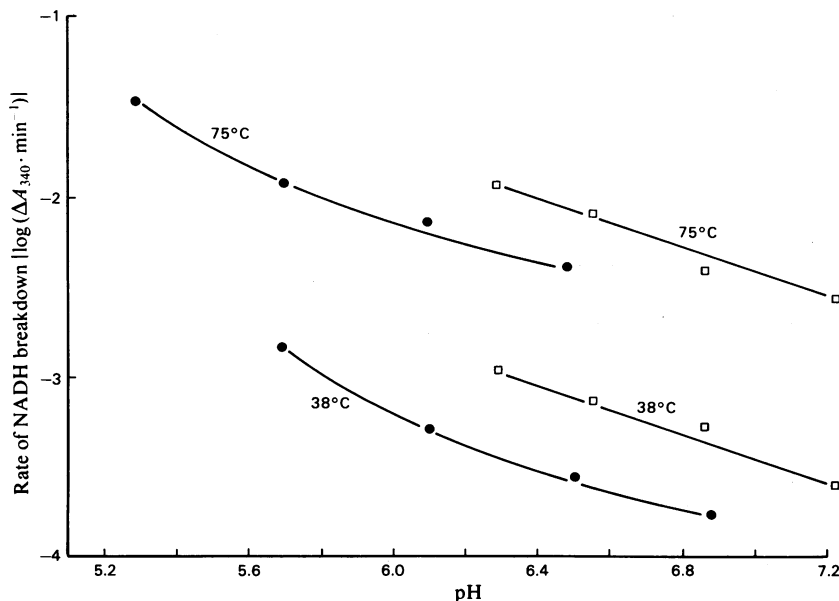


Fig. 1. Effect of pH on NADH stability at 38°C and 75°C

●, in 0.005 M-Mes/NaOH buffer; □, in 0.005 M-KH₂PO₄/Na₂HPO₄ buffer. Ionic strength is 0.01 M in both cases (see the Experimental section).

5.75×10^{-3} litre \cdot mol⁻¹ \cdot cm⁻¹. This decrease is approximately twice that reported by McComb *et al.* (1976).

The effect of pH on NADH stability at 38 and 75°C is shown in Fig. 1.

The effect of temperature on NADH stability is marked. For the buffers and ionic strengths shown in Fig. 1, at pH 6.3 NADH degradation is approx. 35 times faster at 75°C, than at 25°C. At pH 6.3 and an ionic strength of 0.01 M, half the cells NADH would be degraded within about 2.7 h. Under the condition of the assay in 17 mM-Tris/HCl buffer, pH 7.0, at 25°C, NADH degradation is 0.044% per min. At 75°C, the buffer pH is 5.6 and NADH degradation is approx. 2.8% over the 1 min time course of the enzyme assay.

Effect of temperature on $K_3\text{Fe}(\text{CN})_6$

Under the conditions of the enzyme assay, the molar absorption coefficient of $K_3\text{Fe}(\text{CN})_6$ at 420 nm decreased linearly from 20 to 80°C by 0.2% for each 1°C rise in temperature and at 75°C was 0.91×10^3 litre \cdot mol⁻¹ \cdot cm⁻¹. There was no degradation of $K_3\text{Fe}(\text{CN})_6$ in the temperature range 20–80°C.

Verification of the soluble nature of the purified NADH dehydrogenase

Incubation of NADH dehydrogenase with phospholipase A (50 units \cdot ml⁻¹ at pH 7.5 at 37°C for

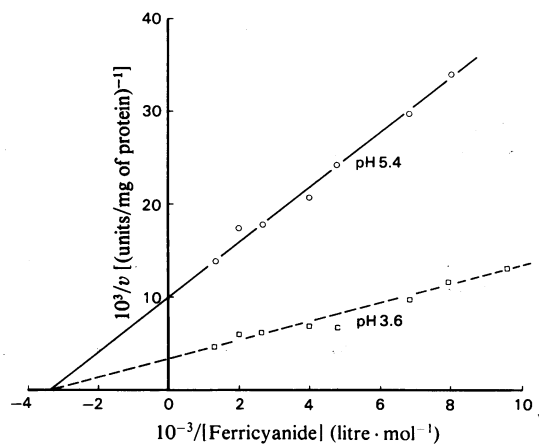


Fig. 2. Effect of pH on the V_{max} and K_m of NADH dehydrogenase

○, in 0.05 M-Tris/HCl, pH 5.4; □, in 0.05 M-succinic acid/KOH, pH 3.7.

20 min) or phospholipase C (4 units \cdot ml⁻¹ at pH 7.5 at 37°C for 20 min), acidification with HCl to pH 5 for 20 min, or addition of NaCl to a final concentration of 2% did not result in precipitation or any loss of activity. These procedures might be expected to precipitate lipoprotein material (Penefsky & Tzagoloff, 1971). The enzyme was purified by

typical protein purification procedures, including Sephadex G-150 chromatography.

Purification and properties

The results of the purification are shown in Table 1. About 25% of the total NADH dehydrogenase activity is soluble. The enzyme was purified 46-fold, with an overall yield of 7%. Coomassie Blue staining of SDS/polyacrylamide electrophoresis gels loaded with 90 μg of purified enzyme showed only one protein band.

The molecular mass of the enzyme, determined by using Sephadex G-150 gel filtration chromatography and SDS/polyacrylamide-gel electrophoresis, was approx. 50 000 Da.

The isoelectric point was determined by isoelectric focusing to be 4.5.

The pH optimum of the enzyme was 3.6, with 70% activity remaining at pH 3.4 and 4.1. Fig. 2 shows that the activity maximum at pH 3.6 is not due to an alteration of the apparent K_m for ferricyanide but is due to an alteration in V_{max} .

The purified enzyme contains, per mol of enzyme, 0.05 mol of FMN (no FAD), 0.16 mol of acid-labile sulphide, and 2.2 mol of iron.

Neither the reduced nor oxidized form of the enzyme (at 0.37 $\text{mg}\cdot\text{ml}^{-1}$) exhibited a detectable e.s.r. signal.

Effect of temperature on activity

The thermostability of the enzyme is shown in Fig. 3. The enzyme retains 80% of its activity on heating at 95°C for 10 min; it is completely denatured by autoclaving [103.5 kPa (15 lbf/in²), 121°C] for 20 min, and this method was used to provide material for non-enzymic controls.

The Arrhenius plots of the soluble and the membrane-bound enzymes are shown in Fig. 4. The soluble enzyme does not exhibit any discontinuity in the Arrhenius plot, whereas the membrane-bound enzyme exhibits two discontinuities, at about 33 and 64°C.

Substrate effects

The apparent K_m for ferricyanide in the presence of 0.29 mM-NADH is 0.29 mM (Fig. 5). The appar-

ent K_m for NADH in the presence of 0.65 mM-ferricyanide is 0.04 mM (Fig. 6). The enzyme is inhibited by high concentrations of NADH and ferricyanide. The apparent K_m for ferricyanide of the membrane-bound enzyme, in the presence of 0.29 mM-NADH, is 0.28 mM (Fig. 5). The membrane-bound enzyme is also inhibited by ferricyanide (Fig. 5), and by high concentrations of NADH (results not shown).

The purified enzyme does not oxidize NADPH or reduce dichloroindophenol.

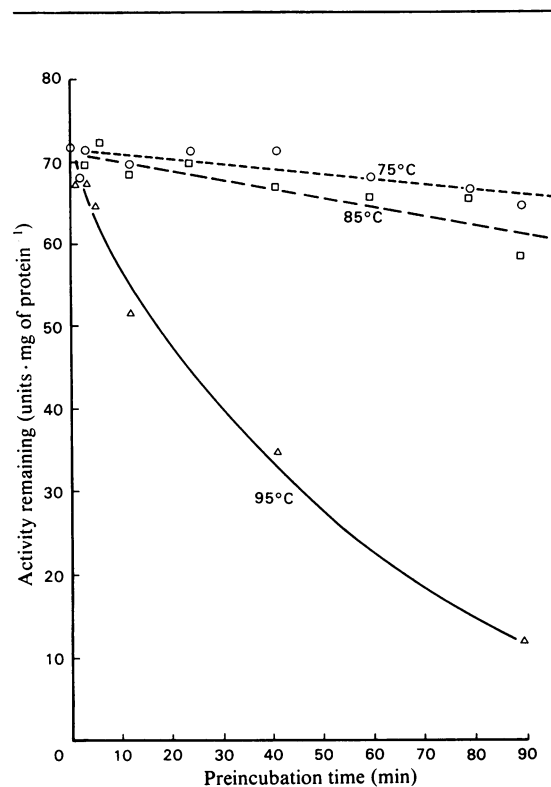


Fig. 3. Thermal denaturation of soluble NADH dehydrogenase

Enzyme samples were preincubated at the temperatures shown and then assayed at 75°C as described in the Experimental section.

Table 1. Summary of purification of NADH dehydrogenase

Purification step	Total activity (units)	Total protein (mg)	Volume (ml)	Specific activity (units · mg ⁻¹)	Recovery (%)	Purification (-fold)
Cell-free extract	20 500	9400	450	2.18	100	—
Suspended membrane particles	13 400	4980	80	2.69	65	—
High speed supernatant	4120	2800	380	1.47	20	1.0
QAE-Sephadex chromatography	2020	312	300	6.47	10	4.4
Gel filtration chromatography	1610	98	25	16.43	8	11.2
Hydroxyapatite chromatography	1375	20	40	68.75	7	46.8

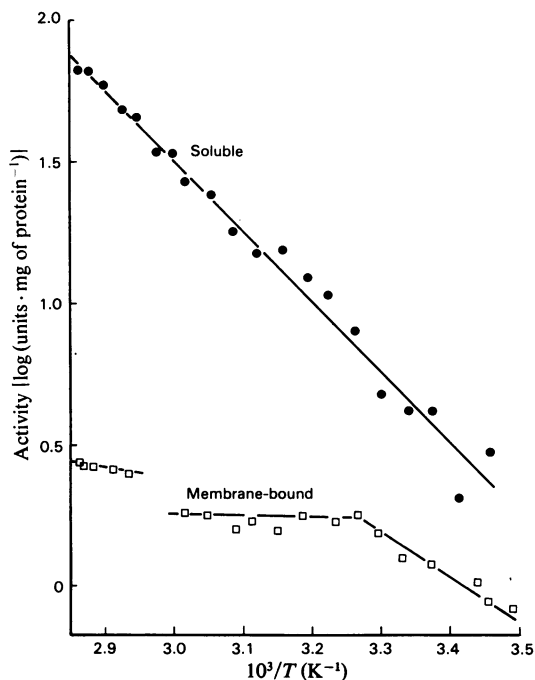


Fig. 4. Effect of temperature on soluble and membrane-bound NADH dehydrogenase activity

The assay was carried out as described in the Experimental section except for a small correction for the change in the molar absorption coefficient of ferricyanide with temperature. Thermal denaturation over the time of the assay was negligible (see Fig. 3). ●, soluble NADH dehydrogenase; □, washed membrane particles prepared as described by Hickey & Daniel (1979).

Inhibitors and activators

Cyanide, azide, *N*-heptyl-4-hydroxyquinoline-*N*-oxide, antimycin A, rotenone and amytal do not inhibit the purified enzyme (Table 2). The enzyme is inhibited by cupferron, Fe^{3+} , Cu^{2+} , and iodoacetic acid, and is activated by $\alpha\alpha'$ -dipyridyl, EDTA and guanidine hydrochloride. Inhibition by cupferron is competitive with respect to ferricyanide. Inhibition by Cu^{2+} may be non-competitive with respect to ferricyanide, since a double-reciprocal plot shows an unchanged K_m for ferricyanide but a decreased V_{\max} ; alternatively, Cu^{2+} may inhibit the enzyme irreversibly. The enzyme is also inhibited by ATP, NAD and FMN, but not by ADP or FAD (Table 2). Inhibition by ATP is competitive with respect to NADH (Fig. 6) but uncompetitive with respect to ferricyanide (Fig. 7). Inhibition by NAD is competitive with respect to ferricyanide (Fig. 7).

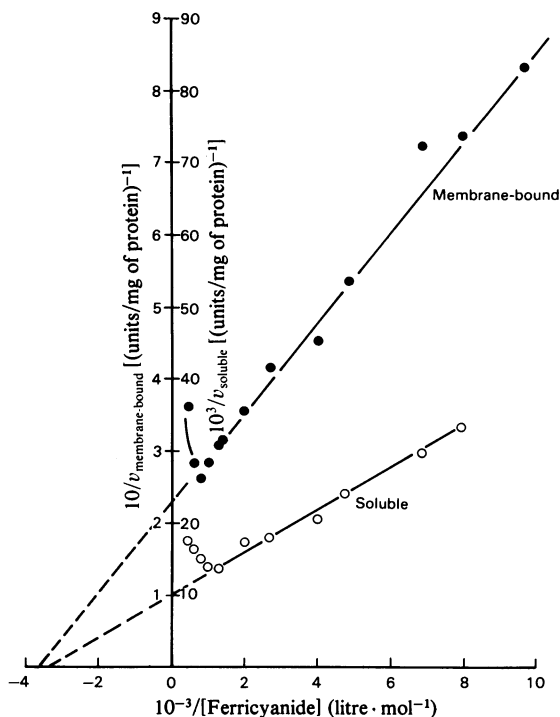


Fig. 5. Effect of ferricyanide concentration on soluble and membrane-bound NADH dehydrogenase activity. Assays were carried out as described in the Experimental section. ○, soluble NADH dehydrogenase; ●, washed membrane particles (prepared as described by Hickey & Daniel, 1979).

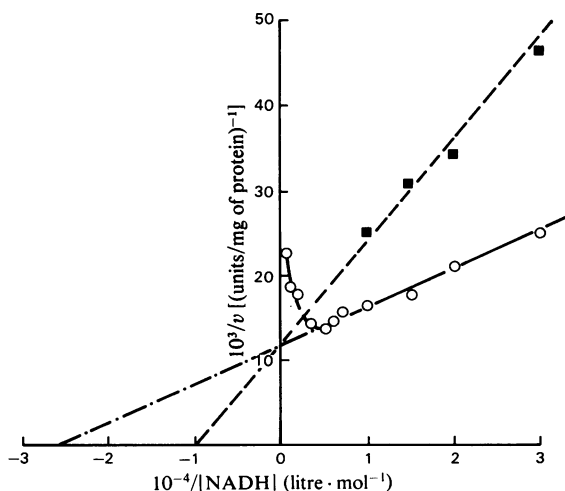


Fig. 6. Effect of NADH concentration on the activity of soluble NADH dehydrogenase in the presence and absence of ATP

Assays were carried out as described in the Experimental section. ○, control; ■, +0.6 mM-ATP.

Table 2. *Inhibitors and activators of NADH dehydrogenase*

The following had no effect: rotenone, $2\mu\text{mol}\cdot\text{mg}$ of protein⁻¹, 10^{-4}M ; *N*-heptyl-4-hydroxyquinoline-*N*-oxide, $0.2\mu\text{mol}\cdot\text{mg}$ of protein⁻¹, 10^{-5}M ; Antimycin A $0.2\mu\text{mol}\cdot\text{mg}$ of protein⁻¹, 10^{-5}M ; sodium azide, 10^{-2}M ; potassium cyanide, 10^{-2}M ; sodium amylal, 10^{-2}M ; sodium barbitone, 10^{-2}M ; *o*-phenanthroline, 10^{-2}M ; 8-hydroxyquinoline, 10^{-4}M ; bathophenanthroline, 10^{-4}M ; trifluoro-1-(2-thienyl)-1,3-butane-dione, 10^{-2}M ; Mg^{2+} , $6.6 \times 10^{-6}\text{M}$; Ni^{+} , $6.6 \times 10^{-6}\text{M}$; Mn^{2+} , $6.6 \times 10^{-6}\text{M}$.

Moderator	Concn. (M)	Activity (%)
Iodoacetic acid	10^{-2}	10
Guanidine hydrochloride	10^{-2}	156
Sodium pyrophosphate	10^{-2}	100
ADP	10^{-3}	100
ATP	10^{-4}	91
	3.30×10^{-3}	43
	8.00×10^{-3}	0
NAD	10^{-4}	83
	8.0×10^{-4}	34
	8.0×10^{-3}	0
FMN	1.5×10^{-4}	50
FAD	10^{-3}	100
$\alpha\alpha'$ -Dipyridyl (in ethanol)	10^{-2}	127
EDTA	10^{-2}	180
Cupferron	10^{-4}	62
Fe^{3+}	6.6×10^{-6}	52
Cu^{2+}	6.6×10^{-6}	20
Zn^{2+}	6.6×10^{-6}	25

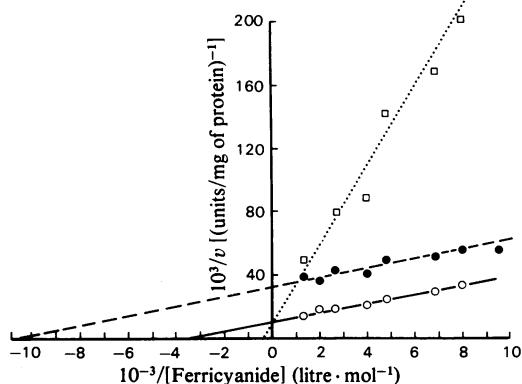


Fig. 7. Effect of ferricyanide concentration on the activity of soluble NADH dehydrogenase in the presence of ATP and of NAD

Assays were carried out as described in the Experimental section. O, control; ●, +3.3 mM-ATP; □, +0.8 mM-NAD.

Discussion

The soluble NADH dehydrogenase prepared from *T. aquaticus* strain T-351 by physically disrupting

the cell was in true solution and was not a lipoprotein.

The molecular mass of the enzyme, about 50 000 Da, is typical of NADH dehydrogenases isolated from micro-organisms (e.g. Mains *et al.*, 1980; Thomson & Shapiro, 1981; Duncan & Mackler, 1966; DerVartanian, 1972), and falls into the low-molecular-weight category of such enzymes.

The double-substrate-inhibition displayed by the soluble and membrane-bound enzymes is evidence in favour of the intact nature of the soluble enzyme, and is in contrast to the behaviour of many low-molecular-weight NADH dehydrogenases. For example, the enzyme of Dooijewaard & Slater (1976*a,b*) did not display this double-substrate inhibition (double substrate inhibition was displayed in the high-molecular-weight form of their enzyme however, and was presumed to be due to competition by the substrates for access through a single cleft to the active centres of the enzyme). This, and the similarity between the K_m for ferricyanide of the soluble enzyme (0.29 mM) and the membrane-bound enzyme (0.28 mM), suggests that the soluble enzyme is derived from the membrane-bound complex.

FMN appears to be a prosthetic group of the enzyme. This is not unexpected, for although FAD is a common prosthetic group of other mesophilic bacterial dehydrogenases (e.g. Dancey, 1976), it is hydrolysed at high temperature (Yagi & Tada, 1960). Although the prosthetic group of the NADH dehydrogenase of the thermophile *B. stearothermophilus* is also thought to be FMN (Mains *et al.*, 1980), the enzyme from the extreme thermophile *B. caldotenax* contains FAD (Kawada *et al.*, 1981). Both the FMN and labile sulphide contents of our purified enzyme are very low. The ratio of FMN, iron and labile sulphide per mol of enzyme is 0.049:2.2:0.16. This ratio in other low-molecular-weight enzyme preparations is typically 1:4:4 (e.g. Hatefi & Stempel, 1969), but other low ratios have been reported (Duncan & Mackler, 1966; Ramnarayanan *et al.*, 1971). The 2.2 mol of iron found, and the absence of any e.s.r. signal at liquid- N_2 temperatures, suggest the presence of a single [4Fe-4S] cluster with much of the acid-labile sulphur being lost during handling. Most FMN is also lost, but much of the iron remains bound to the apoprotein. These losses are unexpected in view of the mild methods employed for preparation and purification, and we cannot rule out the possibility that the enzyme lacks both a flavin coenzyme and an iron-sulphur centre, and that the iron content is due to adsorbed contamination.

The low pH optimum is very rare among NADH dehydrogenases, and is unusual in that the effect is due to an alteration of V_{max} and not K_m .

The enzyme exhibits double-substrate inhibition, strongly suggesting that NADH and ferricyanide

compete for binding to the active site. NAD inhibition of the enzyme is competitive with respect to ferricyanide, confirming this.

The enzyme is inhibited by physiological concentrations of ATP, allowing control of electron transport by ATP concentration. Inhibition by ATP is competitive with respect to NADH, whereas it is uncompetitive with respect to ferricyanide. This could be explained if ATP binds to an enzyme-ferricyanide binary complex, but not to an enzyme-NAD(H) binary complex, and if NADH and ferricyanide compete for the same binding site, as suggested above.

The inhibition by high concentrations of FMN is probably non-physiological. Cupferron inhibits the enzyme competitively with respect to ferricyanide, suggesting that the site of ferricyanide reduction contains iron. However, *o*-phenanthroline does not inhibit activity. The activation effect of $\alpha\alpha'$ -dipyridyl and EDTA suggests that the enzyme is in a state of inhibition by metal ions. This is supported by the finding that micromolar concentrations of copper, iron and zinc inhibit activity. The mode of inhibition by copper may be noncompetitive and the action of $\alpha\alpha'$ -dipyridyl appears to result from the removal of a noncompetitive inhibitor (results not shown). The low pH optimum for the enzyme may be due to competition by H^+ with noncompetitively inhibiting metal ions (Tipton & Dixon, 1979).

The NADH dehydrogenase from *T. aquaticus* strain T-351 is very thermostable, having a half-life at 95°C of 35 min. As was observed by Kawada & Nosoh (1981) for *B. caldotenax*, the temperature-activity relationship of the membrane-bound and soluble forms of the enzyme differed. Our membrane-bound enzyme exhibited discontinuities in the Arrhenius plot, corresponding approximately to the membrane phase transition temperatures at 39 and 66°C (Jansen *et al.*, 1982), whereas the soluble enzyme exhibited no discontinuity, reflecting its lipid-free environment.

References

- Amelunxen, R. & Lins, M. (1968) *Arch. Biochem. Biophys.* **125**, 765-769
- Bates, R. G. (1962) *J. Res. Natl. Bureau Standards* **66A**, 179
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248-254
- Brumby, P. E. & Massey, V. (1967) *Methods Enzymol.* **10**, 463-474
- Burch, H. B. (1957) *Methods Enzymol.* **3**, 960-962
- Dancey, G. F. (1976) *Diss. Abstr. Int. Ser. B* **37**, 737
- DerVartanian, D. V. (1972) *Z. Naturforsch.* **27B**, 1082-1084
- Dooijewaard, G. & Slater, E. C. (1976a) *Biochim. Biophys. Acta* **440**, 1-15
- Dooijewaard, G. & Slater, E. C. (1976b) *Biochim. Biophys. Acta* **440**, 16-35
- Duncan, H. M. & Mackler, B. (1966) *Biochemistry* **5**, 45-50
- Fee, J. A., Findling, K. L., Lees, A. & Yoshida, T. (1978) in *Frontiers of Biological Energetics* (Dulton, P. L., Leigh, J. S. & Scarpa, A., eds.), vol. 1, pp. 118-126, Academic Press, New York
- Fogo, J. K. & Popowsky, M. (1949) *Anal. Chem.* **21**, 732-734
- Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S. & Singh, R. N. M. (1966) *Biochemistry* **5**, 467-477
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751-766
- Hatefi, Y. & Stempel, K. E. (1969) *J. Biol. Chem.* **244**, 2350-2357
- Hickey, C. W. & Daniel, R. M. (1979) *J. Gen. Microbiol.* **114**, 195-200
- Hirata, H., Sone, N., Yoshida, M. & Kagawa, Y. (1976) *Biochem. Biophys. Res. Commun.* **69**, 665-671
- Hirata, H., Sone, N., Yoshida, M. & Kagawa, Y. (1977) *J. Supramol. Struct.* **6**, 77-84
- Jansen, G. J., Daniel, R. M., Nicholson, B. K. & Morgan, H. W. (1982) *Biochim. Biophys. Acta* **685**, 191-195
- Kawada, N. & Nosoh, Y. (1981) *FEBS Lett.* **124**, 15-18
- Kawada, N., Takeda, K. & Nosoh, Y. (1981) *J. Biochem. (Tokyo)* **89**, 1017-1027
- King, T. E. & Norris, R. O. (1967) *Methods Enzymol.* **10**, 634-641
- Koffler, H. & Gale, G. O. (1957) *Arch. Biochem. Biophys.* **67**, 249-251
- Mains, I., Power, D. M., Thomas, E. W. & Buswell, J. A. (1980) *Biochem. J.* **191**, 457-465
- McComb, R. B., Bond, L. W., Burnett, R. W., Keech, R. C. & Bowers, G. N. (1976) *Clin Chem.* **22**, 141-150
- Penefsky, H. S. & Tzagoloff, A. (1971) *Methods Enzymol.* **22**, 204-219
- Ramanarayanan, M., Appaji Rao, N. & Vaidyanathan, C. S. (1971) *Can. J. Biochem. Biophys.* **8**, 214-218
- Thomson, J. W. & Shapiro, B. M. (1981) *J. Biol. Chem.* **256**, 3077-3084
- Tipton, K. F. & Dixon, H. B. F. (1979) *Methods Enzymol.* **63**, 183-234
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406-4412
- Wisdom, C. & Welker, N. E. (1973) *J. Bacteriol.* **114**, 1336-1345
- Yagi, K. & Tada, M. (1960) *Biochem. Prep.* **7**, 51-56