Multi-enzyme complexes in thermophilic organisms: isolation and characterization of the pyruvate dehydrogenase complex from *Thermus aquaticus* AT62

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The pyruvate dehydrogenase complex from the thermophilic bacterium *Thermus aquaticus* was purified by Triton X-100 extraction and chromatography on phenyl-Sepharose CL-4B and HPLC-hydroxyapatite. The electrophoretic pattern of the purified enzyme complex was similar to that of the enzyme complex from *Bacillus subtilis*, with four bands: the α -chain (M_r 39600) and β -chain (M_r 37500) of the pyruvate dehydrogenase component, the dihydrolipoamide acetyltransferase component (M_r 58500) and the dihydrolipoamide dehydrogenase component (M_r 53900). Antibodies against the purified *T. aquaticus* pyruvate dehydrogenase complex cross-reacted with the enzyme complex from *B. subtilis* and, to a minor extent, with that from bovine heart. No cross-reactivity could be observed with the enzyme complex from *Escherichia coli*. The *T. aquaticus* enzyme complex had a temperature maximum at 72 °C. 2-Oxobutyrates were a poor substrate and other 2-oxoacids were competitive inhibitors of the overall reaction. Long-chain 2-oxoacids showed a greater inhibitory effect, possibly caused by hydrophobic interactions. GTP inhibited the enzyme activity. Regulation of the pyruvate dehydrogenase complex from *T. aquaticus* by allosteric mechanisms or by reversible phosphorylation could not be demonstrated.

Introduction

Although thermostable enzymes from thermophilic organisms have been thoroughly investigated (Argos *et al.*, 1979; Jaenicke, 1981; Zuber, 1981), little is known about the special features of multi-enzyme complexes from such organisms. Henderson *et al.* (1979) showed that the pyruvate dehydrogenase complex from *Bacillus stearothermophilus* appeared to be similar in structure to that from mesophilic organisms (Packman *et al.*, 1988).

Pyruvate dehydrogenase complexes can be subdivided into two classes. In the type I pyruvate dehydrogenase complex from Gram-negative bacteria, the 24 identical subunits of dihydrolipoamide acetyltransferase (E2, EC 2.3.1.12) form a cubic core. The 24 identical subunits of the pyruvate dehydrogenase component (E1, EC 1.2.4.1) bind to the edges of this cube in an octahedral symmetry, while 12 subunits of the dihydro-

lipoamide dehydrogenase component (E3, EC 1.8.1.4) are attached to its faces. The type II pyruvate dehydrogenase complex, which is found in Gram-positive bacteria and in eukaryotes, possesses a similar structure with an E2 core surrounded by subunits of the E1 and E3 components. There are, however, 60 subunits of both the E1 and the E2 components, so that the complex has an icosahedral symmetry. The subunits of the E1 component are formed from two non-identical polypeptide chains (α and β). Component X, whose function is still unknown, is found in eukaryotes. In higher organisms regulatory enzymes, catalysing reversible phosphorylation of the E1 component, are also bound to the enzyme complex (Reed, 1974, 1981; Wieland, 1983; Yeaman, 1989).

Here we report the isolation and characterization of a type II pyruvate dehydrogenase complex from the thermophilic bacterium *Thermus aquaticus*.

Methods

Growth of bacteria. *Thermus aquaticus* AT-62 (DSM 674) was obtained from the Deutsche Sammlung für Mikroorganismen, Braunschweig, Germany. Three different media were used for cell

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cultivation. A medium containing 0.8% proteose peptone, 0.4% yeast extract, and 0.3% NaCl in distilled water (Oshima & Imahori, 1974), and a minimal medium (Yoshida *et al.*, 1984) supplemented with 0.2% sodium pyruvate were used for 250 ml cultures grown in gently shaken flasks at 70 °C for 15 h. A semi-synthetic medium, M162 (Degryse *et al.*, 1978), supplemented with 0.2% sodium pyruvate, was used in a 20 litre Intensor 20b fermenter (Giovanna Frères, Monthy, Switzerland); cultures were grown for 15 h at 70 °C with 0.2 vols vol.⁻¹ min⁻¹ air and stirring (1200 r.p.m.).

Crude extracts were obtained by sonication of a suspension of the centrifuged cells in 2 vols 50 mM-potassium phosphate, pH 7.5, 0.1 mM-EDTA, 1 mM-PMSF, 2 mM-DTT, 3 mM-MgCl₂ and 5 mM-β-mercaptoethanol, for 4 min at 50 W at 4 °C, using a type B-12 sonicator (Branson). Cell debris was removed by centrifugation at 38000 g for 30 min.

Enzyme assays and protein determination. The overall activity of the pyruvate dehydrogenase complex was measured according to Schwartz & Reed (1970), and the activity of the E3 component according to Schmincke-Ott & Bisswanger (1981). The reduction of NAD was in both cases followed photometrically at 340 nm at 70 °C for the *T. aquaticus* enzymes and at 30 °C for the enzymes from other sources. The enzyme activities were given in nkat (nmol NADH produced s⁻¹).

Protein concentration. Protein concentration was determined according to a modified Lowry method (Hartree, 1972). The BCA protein assay reagent (Pierce) was used for purified enzyme samples.

Purification of the pyruvate dehydrogenase complex. Triton X-100 extraction. The crude extract was warmed to room temperature and Triton X-100 was added to a final concentration of 20% (v/v). The mixture was stirred for 10 min, then centrifuged at 100000 g at room temperature for 30 min. The clear yellow lower layer containing the enzyme activity was carefully removed by a syringe through the upper layer which contained the Triton X-100.

Hydrophobic chromatography. A 2 × 16 cm phenyl-Sepharose CL-4B column was equilibrated with 1 M-potassium phosphate, pH 7.0, 0.1 mM-DTT, 0.1 mM-EDTA. DTT, EDTA and 2 M-potassium phosphate, pH 7.0 were added to the Triton X-100 extract to give a final concentration of 1 M-potassium phosphate, pH 7.0, 0.1 mM-DTT, 0.1 mM-EDTA. The column was run with a flow rate of 2 ml min⁻¹ with an FPLC pump system (Pharmacia-LKB). A 150 ml gradient of 1 to 0.01 M-potassium phosphate, pH 7.0, 0.1 mM-DTT, 0.1 mM-EDTA was applied and 5 ml fractions were collected. Active fractions were pooled and layered carefully onto the same volume of 35% (w/v) sucrose in 50 mM-potassium phosphate, pH 7.0, in an ultracentrifuge tube and centrifuged for 4 h at 176000 g and 4 °C. The clear yellow pellet was dissolved in a small volume of 25 mM-potassium phosphate, pH 7.0.

Adsorption chromatography. The enzyme was applied to a 7.8 × 100 mm Bio-Gel HPHT hydroxyapatite column, equilibrated with 25 mM-potassium phosphate, pH 7.0, 0.2 mM-DTT, and eluted at a flow rate of 0.1 ml min⁻¹. For each run 1 mg protein was applied to the column in 1 ml, and the process was repeated in order to purify more protein. Elution was with a 10 ml linear gradient of 25 to 500 mM-potassium phosphate pH 7.0, 0.2 mM-DTT, and 0.2 ml fractions were collected.

Electrophoresis. SDS-PAGE (Laemmli, 1970) was used with a gradient of 10 to 20% (w/v) acrylamide. Staining of protein bands was with Coomassie brilliant blue R-250 (Fairbanks *et al.*, 1971).

Sucrose gradient centrifugation. The enzyme preparation (50 µl) was layered on to a linear 5.2 ml gradient from 10 to 50% (w/v) sucrose in 0.05 M-MOPS/HCl, pH 7.0, and centrifuged in a SW 65 Ti swinging bucket rotor (Beckman) at 145000 g and 4 °C for 3 h (Martin & Ames, 1961).

Electron microscopy. The samples were negatively stained with uranyl oxalate according to Mellema *et al.* (1967) on copper grids (mesh 400, Veco, Solingen, Germany), and examined with an EM109 electron microscope (Zeiss).

Isolation of rabbit antibodies. The antigen (0.8 mg protein in 1 ml 50 mM-MOPS/HCl, pH 7.0, emulsified in 1 ml of complete Freund's adjuvant by sonication for some seconds) was injected subcutaneously into a rabbit. After 2 and 3 weeks, two further injections were given of 0.2 mg antigen in 0.5 ml MOPS/HCl, pH 7.0, emulsified in 0.5 ml incomplete Freund's adjuvant. Blood (ca. 10 ml) was taken 28 d after the first injection. The immunoglobulin fraction was prepared from serum by affinity chromatography on a 1 × 5 cm Protein A-Sepharose CL-4B column, equilibrated with 0.1 M-potassium phosphate, pH 8.0.

Labelling with ³²P. Cells of *T. aquaticus*, grown in 250 ml of medium M162, supplemented with 92.5 MBq ³²P_i, at 70 °C for 8 h, were collected by centrifugation at 10000 g for 10 min. After sonication the crude extract was centrifuged at 38000 g for 30 min. Samples containing 0.1 µg protein each were applied to a 10 to 15% (w/v) gradient acrylamide gel and run in a PhastSystem electrophoresis apparatus (Pharmacia-LKB). After silver staining according to Heukeshoven & Dernick (1985) the gel was dried for 2 h at 70 °C. Kodak X-OMAT TM AR film was exposed to the gel for 15 d with a Kodak X-Omatic regular intensifying screen at room temperature.

Chemicals. Proteose peptone no. 3, yeast extract and complete and incomplete Freund's adjuvant were purchased from Difco; sodium pyruvate and other media and buffers were from Merck; 1,4-dithiothreitol (DTT), phenylmethylsulphonyl fluoride (PMSF), and substrates and cofactors for the enzyme tests were from Boehringer Mannheim; acrylamide, Coomassie brilliant blue R-250, glyoxylic acid, *N,N'*-methylene-bis-acrylamide, MOPS, SDS and Triton X-100 were from Serva; 2-oxobutyrate, 2-oxo-n-valerate and 2-oxo-n-caprylate were from Fluka and sodium 2-oxocaproate was from Sigma. Phenyl-Sepharose CL-4B and Protein A-Sepharose CL-4B were obtained from Pharmacia-LKB; the Bio-Gel HPHT column was from Bio-Rad; ³²P_i was from Amersham.

Results

Induction of the pyruvate dehydrogenase activity in T. aquaticus

To test whether in *T. aquaticus* biosynthesis of the pyruvate dehydrogenase complex is induced by pyruvate this organism was grown on the three different media all supplemented either with 0.2% sodium pyruvate or glucose. The largest cell yields and highest enzyme activities were obtained with the semi-synthetic medium (Table 1). Pyruvate induced the enzyme activity in all three media, but most efficiently in M162 medium (Table 1). The dependence of the enzyme activity on the concentration of pyruvate or glucose in M162 medium was tested. The activity increased to a plateau above 0.4% for pyruvate, which was double that for glucose.

Purification and molecular mass determination of the pyruvate dehydrogenase complex from T. aquaticus

The purification of pyruvate dehydrogenase from *T. aquaticus* was difficult because of the great instability of

Table 1. Cell yields and pyruvate dehydrogenase activities after cultivation of *T. aquaticus* AT62 on different media, supplemented with various carbon sources

The bacteria were grown in 500 ml of culture medium at 70 °C. After 15 h the cells were collected by centrifugation. The overall activity of the pyruvate dehydrogenase complex was measured in the crude extract.

Culture medium	Carbon source (0.2%)	Cell yield (g)	Enzyme activity [nkat (mg protein) ⁻¹]
Complete medium	Pyruvate	0.5	0.70
	Glucose	0.6	0.52
Semi-synthetic medium M162	Pyruvate	1.8	3.9
	Glucose	2.0	2.34
	Glutamate	1.0	0.47
	Glycerol	0.8	0.23
	Acetate	1.1	0.48
Minimal salt medium	Succinate	1.2	0.20
	Pyruvate	0.96	1.42
	Glucose	1.05	1.23

Table 2. Purification of the pyruvate dehydrogenase complex from *T. aquaticus*

Purification step	Enzyme activity					
	Vol. (ml)	Protein (mg)	Total (nkat)	Specific [nkat (mg protein) ⁻¹]	Yield (%)	Purification factor
Crude extract	28	420	78.3	0.196	—*	—*
Triton X-100 extraction	24	276	88.1	0.319	100	1
Phenyl-Sepharose CL-4B chromatography	30	121.5	80.6	0.663	91.5	2.08
Ultracentrifugation	1	2.1	36.8	17.52	41.8	54.9
Hydroxyapatite chromatography	1.6	0.34	31.3	93.02	35.3	291.6

* Because of perturbations in the crude extract the enzyme activity could not be determined exactly.

the enzyme complex. Small amounts of purified enzyme complex in low yield could be obtained by running the chromatography columns at high speed with an FPLC pump system. The purification steps are summarized in Table 2. A Triton X-100 extraction was followed by application to a hydrophobic phenyl-Sepharose CL-4B column, where the enzyme remained tightly bound but was released at low ionic strength. Concentration and further purification was achieved by ultracentrifugation. After adsorption chromatography on hydroxyapatite a pure enzyme preparation was obtained.

In SDS-PAGE, the purified enzyme complex from *T. aquaticus* gave four bands (Fig. 1, lane 3), similar to those of the enzyme complex from *Bacillus subtilis* (lane 4). In contrast, the bovine heart complex (lane 2) showed five bands with different migration behaviour. The order

of the components is the same for both the mammalian and the bacterial pyruvate dehydrogenase complexes (Barrera *et al.*, 1972; Visser *et al.*, 1980), with the exception of component X, which appeared just below the E3 component in the case of the bovine enzyme complex (DeMarcucci & Lindsay, 1985). For *T. aquaticus* the identity of the upper band with the E2 component was established by the reaction of its biotinylated lipoyl residues with streptavidin, while the second electrophoretic band comigrated with a preparation of the purified free E3 component (not shown).

For the different subunits of the pyruvate dehydrogenase complex from *T. aquaticus* the following M_r values were determined from the migration distances in SDS-PAGE in relation to reference proteins: E1 α , 39 600; E1 β , 37 500; E2, 58 500; E3, 53 900.

The M_r of the native pyruvate dehydrogenase complex from *T. aquaticus* was determined by ultracentrifugation for 3 h in a linear 10 to 50% sucrose gradient at 145 000 *g* with the enzyme complexes from *E. coli* and bovine heart as references. The *T. aquaticus* enzyme complex sedimented faster than the enzyme complexes from both the other organisms, and an M_r value of 11.3×10^6 was calculated (Martin & Ames, 1961). Similar results were obtained by gel-filtration on a FPLC Superose 6 column, where the *T. aquaticus* enzyme complex migrated faster than both the bovine and the *E. coli* complexes and an M_r value of 9.1×10^6 was determined.

The pyruvate dehydrogenase complex from *T. aquaticus* was negatively stained with uranyl oxalate (Fig. 2) under conditions that favoured disaggregation. Only the relatively stable E2 core remained intact, and showed a regular hexameric ring structure.

Immunological cross-reactivity

The electrophoretic pattern of the pyruvate dehydrogenase complex from *T. aquaticus* resembled that of type II, as found in Gram-positive bacteria and eukaryotic organisms. This was confirmed by immunotitration with polyclonal rabbit antibodies against the purified *T. aquaticus* enzyme complex. These antibodies were able to inactivate not only the pyruvate dehydrogenase complex from *T. aquaticus* but equally that from *B. subtilis* and, with rather less efficiency, that from bovine heart. No cross-reactivity, however, was observed with the pyruvate dehydrogenase complex from *E. coli* (Fig. 3).

Kinetic characterization of the *T. aquaticus* enzyme complex

The catalytic activity of the pyruvate dehydrogenase complex from *T. aquaticus* increased with the

Fig. 1. SDS-PAGE of purified pyruvate dehydrogenase complexes from *T. aquaticus* (lane 3), beef heart (lane 2) and *B. subtilis* (lane 4). The order of components of the *B. subtilis* enzyme complex is indicated on the right. Lane 1 shows the reference proteins: bovine serum albumin (BSA), diaphorase from pig heart, and lactate dehydrogenase (LDH) from pig muscle. A total of 1.5 µg of each protein was applied onto the gel.

temperature up to a maximum at 72 °C. This increase gave no simple linear pattern in the Arrhenius plot; rather it appeared to consist of two linear parts corresponding to activation energies of about 80 kJ mol⁻¹ in the lower (<40 °C) and 58 kJ mol⁻¹ in the upper (>50 °C) temperature range (Fig. 4a). Beyond the temperature maximum the activity of the enzyme complex steeply declined and it became completely inactivated above 90 °C. With respect to pH values the overall activity of the pyruvate dehydrogenase complex

showed a broad optimum between pH 5 and 8 with a maximum at pH 7.2 (Fig. 4b).

Besides pyruvate the pyruvate dehydrogenase complex from *T. aquaticus* accepted only 2-oxobutyrate as a substrate. The Michaelis constant for 2-oxobutyrate is tenfold higher and the maximum velocity of its conversion one-third of those for pyruvate (Table 3). Substrate inhibition was observed for both compounds at concentrations tenfold higher than the K_m value. Table 3 also shows the Michaelis constants for NAD and

Fig. 2. Electron micrograph of the purified pyruvate dehydrogenase complex from *T. aquaticus*, negatively stained with uranyl oxalate. The bar corresponds to 20 nm. The arrow shows an intact E2 core.

coenzyme A. In all cases, simple Michaelis-Menten behaviour was observed. As with the pyruvate dehydrogenase complex from other sources and other thiamin-diphosphate-dependent enzymes complete removal of this cofactor caused irreversible loss of enzyme activity. About 18% of the cofactor remained tightly bound to the enzyme complex even after extensive dialysis. The K_m value for thiamin diphosphate in Table 3 was, therefore, corrected for this residual amount of bound cofactor.

2-Oxoacids other than pyruvate and 2-oxobutyrates were competitive inhibitors of the *T. aquaticus* pyruvate dehydrogenase complex with respect to pyruvate. The inhibition decreased with increasing chain length (Table 3). With long-chain 2-oxoacids, e.g. 2-oxocaproate, however, the enzyme complex showed a remarkably strong non-competitive inhibition, which exceeded the competitive inhibition (not shown).

Regulation of the pyruvate dehydrogenase complex from *T. aquaticus*

Mammalian pyruvate dehydrogenase is regulated by reversible phosphorylation (Reed, 1981). Pre-incubation

Fig. 3. Immunotitration of purified pyruvate dehydrogenase complexes from *T. aquaticus* (●), *B. subtilis* (○), *E. coli* (□) and bovine heart (■) with polyclonal rabbit antibodies against the purified pyruvate dehydrogenase complex from *T. aquaticus*, and of pyruvate dehydrogenase complex from *T. aquaticus* with rabbit control serum (▲). The enzyme preparations (50 μg each) were incubated together with the antibodies in a total volume of 0.5 ml 0.1 M-potassium phosphate, pH 7.6, at 20 °C for 30 min, and for further 90 min at 4 °C. The overall activity of the pyruvate dehydrogenase complex was determined in the supernatant after centrifugation of the samples at 12000 r.p.m. for 5 min. 100% corresponds to an enzyme activity of 5.5 nkat ml⁻¹.

Table 3. Kinetic constants of the pyruvate dehydrogenase complex from *T. aquaticus*

Compound	K_m (M)	V (nmol s ⁻¹)	K_i
Pyruvate	3.9×10^{-4}	8.2	—
2-Oxoglutarate	4.1×10^{-3}	2.5	—
Thiamin diphosphate	0.2×10^{-6}	ND	—
Coenzyme A	9.2×10^{-6}	ND	—
NAD	7.3×10^{-5}	ND	—
Glyoxylate	—	—	6.5×10^{-4}
2-Oxo-n-valerate	—	—	6×10^{-3}
2-Oxo-n-caproate	—	—	34×10^{-3}

ND, Not determined.

of crude extract from *T. aquaticus* (30 mg protein ml⁻¹) with 1 mM-ATP in the presence of 1.2 mM-MgCl₂, 0.1 mM-EDTA, 0.1 mM-EGTA, 2 mM-DTT in 50 mM-MOPS/HCl, pH 7.0, at 70 °C, however, caused no change in the pyruvate dehydrogenase activity. When the enzyme complex was pre-incubated with GTP instead of ATP time-dependent inhibition was observed, which reached a plateau at 51% (0.5 mM-GTP) and 67% (1 mM-GTP) inhibition after 10 min. This inhibition could be partially reversed by gel-filtration on a Superose 6 column (Table 4), which makes covalent modification unlikely, though reactivation of the enzyme complex by a complex-bound phosphatase during gel-filtration could

Fig. 4. Effect of temperature (a) and pH (b) on the enzymic activity of the pyruvate dehydrogenase complex from *T. aquaticus*. The overall activity of the enzyme complex was tested with 30 µg enzyme per ml of test mixture, 2.0 mM-pyruvate in 0.1 M-potassium phosphate, pH 7.6. For the pH curve the buffer system of Teorell & Stenhagen (1938) was applied instead of potassium phosphate; the temperature was 70 °C.

Table 4. Gel-filtration of GTP-inactivated pyruvate dehydrogenase complex from *T. aquaticus*

Samples of crude extract of *T. aquaticus* (3 mg protein) were incubated with different amounts of GTP in 50 mM-MOPS/HCl, pH 7.0, 1.2 mM-MgCl₂, 0.1 mM-EDTA, 0.12 mM-EGTA, 2 mM-DTT in a total volume of 0.5 ml at 70 °C for 25 min. Then the samples were applied to a Superose 6 column equilibrated with MOPS/HCl, pH 7.0, and connected with an FPLC system. The active fractions were pooled and tested after elution.

GTP concentration in incubation assay (mM)	Pyruvate dehydrogenase activity			
	After pre-incubation		After gel-filtration	
	nmol s ⁻¹	%	nmol s ⁻¹	%
0	5.8	100	4.2	100
0.5	3.1	53	3.7	88
1.0	2.2	38	3.6	86

not be completely ruled out. To test this *T. aquaticus* was grown for 8 h on medium M162 in the presence of ³²P_i. The crude extract after Triton X-100 extraction was applied to SDS-PAGE and an autoradiographic film was exposed to the gel. After exposure for 15 d bands appeared on the film in the upper *M_r* range above 68 000. However, no darkening could be detected in the *M_r* range between 36 000 and 60 000, indicating that there was no phosphorylation of the subunits of the pyruvate dehydrogenase complex.

Discussion

The subunit structure of the E1 component, the high apparent *M_r* of the native enzyme complex, and the symmetry of the E2 core as derived from electron micrographs [quite similar to the E2 component from *B. stearothermophilus*, yeast or mammals (Henderson *et al.*, 1979; Junger *et al.*, 1973; Bleile *et al.*, 1981)] clearly

showed that the pyruvate dehydrogenase complex of *T. aquaticus* belongs to type II. If it can be taken as a general rule that type I complexes are limited to Gram-negative bacteria, while Gram-positive bacteria and eukaryotes possess type II complexes, our finding supports the classification of *T. aquaticus* as a Gram-positive bacterium (Hensel *et al.*, 1986), in contrast to its previous description as a Gram-negative bacterium (Brock & Freeze, 1969). Though structural features of enzymes are only a crude tool for phylogenetic classification of organisms, they have been successfully applied in other cases, e.g. with citrate synthase and succinate thiokinase, where distinct structural forms can be related to Gram-negative bacteria on the one hand and Gram-positive bacteria and eukaryotes on the other hand (Weitzman & Jaskowska-Hodges, 1982).

The close relationship between the type II complexes is confirmed by the cross-reactivity of the pyruvate dehydrogenase complexes from *T. aquaticus* and *B. subtilis* and the lack of any immunological reaction with the type I enzyme complex from *E. coli*. The clear cross-reactivity between the bovine and the *T. aquaticus* enzyme complexes is striking. Comparison of pyruvate dehydrogenase complexes from different organisms, however, has revealed large species-specific differences among the E1 and the E2 components, while the E3 component is highly conserved (Guest, 1978), and may be responsible for the immunological cross-reactivity between far-distant species like *Thermus* and cattle.

The regulation of biosynthesis of the pyruvate dehydrogenase complex in *T. aquaticus* seems to be similar to that reported for *E. coli* (Dietrich & Henning, 1970). High enzyme activities were found with pyruvate, an internal inducer of the *ace* operon in *E. coli*, activities were low with acetate and other carbon sources such as glutamate, succinate and glycerol, while growth on glucose yielded intermediate values. Thus, the pyruvate dehydrogenase complex from *T. aquaticus*, like that from *E. coli* (Engel-Rae & Henning, 1973) seems not to be sensitive to catabolite repression. Accumulation of

pyruvate rather than direct induction may be responsible for the effect of glucose. In the complete medium the induction of enzyme activity by pyruvate is less pronounced because of the presence of different metabolites which may influence the regulation of biosynthesis of the enzyme complex.

The relative instability of the *T. aquaticus* pyruvate dehydrogenase complex compared with those from mesophilic bacteria and eukaryotes is remarkable. Proteolytic degradation cannot be ruled out, though EDTA and PMSF as protease inhibitors were present during the purification procedure. Pyruvate dehydrogenase complexes from other sources were subject to proteolytic attack, especially on the E2 component. The characterized degradation products partly retained their enzymatic activity (Gebhardt *et al.*, 1978; Kresze & Ronft, 1980), but no such degradation products of the *T. aquaticus* enzyme complex were found. Dissociation of the enzyme complex into its components may also cause inactivation. Dissociation of subunits of the E3 component occurs during chromatographic steps and ultracentrifugation (Lehmacher & Bisswanger, 1988). Addition of the E3 component to preparations of the enzyme complex, however, caused only a slight recovery of the overall activity.

The instability of the *T. aquaticus* pyruvate dehydrogenase complex is also reflected by its poor temperature stability. While *T. aquaticus* itself is able to grow at temperatures higher than 80 °C, its pyruvate dehydrogenase complex becomes considerably inactivated above 70 °C. Since three enzyme components are involved in the overall reaction, the thermal behaviour of the complex is dictated by the most labile one. Both the E1 and the E2 components are somewhat labile, whereas the E3 component proved to be relatively stable (Lehmacher & Bisswanger, 1988).

A biphasic behaviour pattern with a transition at about 45 °C is observed with various thermophilic enzymes, including the E3 component isolated from *T. aquaticus* (Lehmacher & Bisswanger, 1988). Thermophilic enzymes must maintain their native structure over a very broad temperature range, and the transition temperature between the two phases coincides with the temperature maximum of many mesophilic enzymes. The biphasic behaviour may be an adaption of the enzyme to extreme temperature conditions, with a transition to a metastable conformation in the upper temperature range (Nickerson, 1973; Privalov, 1979; Jaenicke, 1981). For the pyruvate dehydrogenase complex it must be borne in mind that the temperature profile of the overall reaction contains contributions of the different enzyme components and cannot, therefore, be treated in a simple way.

The regulation of the pyruvate dehydrogenase com-

plex of Gram-positive bacteria is not yet well understood. As it is a key enzyme, connecting the glycolytic chain with the citric acid cycle its activity should be subject to strict control. The pyruvate dehydrogenase complex from Gram-negative bacteria like *E. coli* and *Salmonella typhimurium* is controlled allosterically (Bisswanger, 1984), and the enzyme complexes from higher organisms are regulated by reversible phosphorylation of their E1 component (Reed, 1981). Neither of these regulatory mechanisms could be detected in Gram-positive bacteria. This was also the case with the pyruvate dehydrogenase complex from *T. aquaticus*. The only regulatory mechanism observed so far is an inhibition by GTP. This occurred, however, at relatively high concentrations.

The substrate specificity of the pyruvate dehydrogenase complex from *T. aquaticus* is similar to that of the mammalian and the *E. coli* complexes (Blass & Lewis, 1973; Bisswanger, 1981; Pettit & Reed, 1982). Pyruvate and 2-oxobutyrate are the only substrates accepted. Glyoxylate and higher 2-oxoacids act as competitive inhibitors, and their inhibitory effects decrease with increasing chain length. A strong non-competitive inhibition by long-chain 2-oxoacids is observed only with the enzyme complex from *T. aquaticus*. This may result from interactions of the non-polar hydrocarbon chains of the 2-oxoacids with the enzyme surface, which is more hydrophobic in thermophilic proteins than in mesophilic ones (Jaenicke, 1981; Zuber, 1981). Together with thiamin-diphosphate-dependent enzymes from other sources (Morey & Juni, 1968; Bisswanger, 1974; Butler *et al.*, 1977) the *T. aquaticus* pyruvate dehydrogenase complex shares the poorly understood feature, that this cofactor, though being non-covalently bound, cannot be completely removed from the enzyme without destroying its catalytic activity.

In its overall structural features, the pyruvate dehydrogenase complex from *T. aquaticus* shows no significant differences from those of mesophilic organisms that could be attributed to its thermophilic character. Further investigations are projected to explore the adaptation of this enzyme complex to high temperature conditions.

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