NAD⁺-dependent Glyceraldehyde-3-phosphate Dehydrogenase from Thermotoga tenax

THE FIRST IDENTIFIED ARCHAEAL MEMBER OF THE ALDEHYDE DEHYDROGENASE SUPERFAMILY IS A GLYCOLYTIC ENZYME WITH UNUSUAL REGULATORY PROPERTIES

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Nina A. Brunner, Henner Brinkmann, Bettina Siebers, and Reinhard Hensel

From the Department of Microbiology, FB 9, Universität-GH Essen, Universitätstrasse 5, 45117 Essen, Germany and the Department of Cell Biology, Université Paris Sud, 91405 Orsay Cédex, France

The hyperthermophilic archaean Thermotoga tenax possesses two glyceraldehyde-3-phosphate dehydrogenases differing in cosubstrate specificity and phosphate dependence of the catalyzed reaction. NAD⁺-dependent glyceraldehyde-3-phosphate dehydrogenase catalyzes the phosphate-independent irreversible oxidation of D-glyceraldehyde 3-phosphate to 3-phosphoglycerate. The coding gene was cloned, sequenced, and expressed in Escherichia coli. Sequence comparisons showed no similarity to phosphorylating glyceraldehyde-3-phosphate dehydrogenases but revealed a relationship to aldehyde dehydrogenases, with the highest similarity to the subgroup of nonphosphorylating glyceraldehyde-3-phosphate dehydrogenases.

The activity of the enzyme is affected by a series of metabolites. All effectors tested influence the affinity of the enzyme for its cosubstrate NAD⁺. Whereas NADP(H), NADH, and ATP reduce the affinity for the cosubstrate, AMP, ADP, glucose 1-phosphate, and fructose 6-phosphate increase the affinity for NAD⁺. Additionally, most of the effectors investigated induce cooperativity of NAD⁺ binding.

The irreversible catabolic oxidation of glyceraldehyde 3-phosphate, the control of the enzyme by energy charge of the cell, and the regulation by intermediates of glycolysis and gluconeogenesis identify the NAD⁺-dependent glyceraldehyde-3-phosphate dehydrogenase as an integral constituent of glycolysis in T. tenax. Its regulatory properties substitute for those lacking in the reversible nonregulated pyrophosphate-dependent phosphofructokinase in this variant of the Embden-Meyerhof-Parnas pathway.

With deeper insights into their physiology, the Archaea as the descendants of the third ancient lineage in the organismal evolution (1), have proved to be unexpectedly diverse. The central metabolic pathways of carbohydrate catabolism even exhibit a higher variability than found in Bacteria and Eucarya: in addition to modifications of the Entner-Doudoroff pathway (2, 3), several hitherto unknown variants of the classical Embden-Meyerhof-Parnas pathway have been described (4–6).

To address the diversification of the carbohydrate metabolism in Archaea and its regulation in response to growth conditions, we focused on Thermotoga tenax, a hyperthermophilic crenarchaeote able to grow chemolithotrophically as well as chemooorganotrophically. Although a modified Entner-Doudoroff pathway is active in T. tenax, glucose is mainly degraded via a modified Embden-Meyerhof-Parnas pathway that is characterized by a reversible pyrophosphate-dependent phosphofructokinase (7). As an additional peculiarity, T. tenax possesses two pyridine nucleotide-dependent glyceraldehyde-3-phosphate dehydrogenases (GAPDHs) that differ in their cosubstrate specificity (8). Whereas N-terminal sequence features revealed that the NAD⁺-dependent GAPDH is a member of the common phosphorylating GAPDH of Archaea (E.C. 1.2.1.13), the structural affiliation of the NAD⁺-dependent enzyme remained uncertain. An unusually high molecular mass (220 kDa) and the observation that the enzyme also exhibits activity without phosphate suggested a relationship to nonphosphorylating GAPDH (EC 1.2.1.9).

Nonphosphorylating GAPDH (GAPN) has been described as existing in diverse photosynthetic Eucarya such as plants, eucaaryal microalgae, and protists (9–12), as well as in chemoorganotrophic bacteria (13). The enzymes characterized to date catalyze the irreversible oxidation of D-glyceraldehyde 3-phosphate (D-GAP) to 3-phosphoglycerate and show a high specificity for NADP⁺ as cosubstrate. Due to the reaction catalyzed and the inhibition observed in the presence of intermediates of the oxidative pentose phosphate cycle and phosphohydroxy-pyruvate, an intermediate of the serine biosynthesis, it has been concluded that the enzymes fulfill mainly biosynthetic purposes by supplying NADPH for anabolic reactions or in serine biosynthesis (10, 13, 14). Sequence analyses of the genes encoding GAPN of pea and maize, as well as of the bacterium Streptococcus mutans, indicated that GAPN enzymes are not related to phosphorylating GAPDH at all, but belong to the superfamily of aldehyde dehydrogenases (ALDHs) (EC 1.2.1.3), which are characterized by varying degrees of substrate specificity (14, 15). Therefore, despite a similar catalytic mechanism, i.e., hydride transfer via a hemithioacetal intermediate, independent evolutionary origins of phosphorylating and nonphosphorylating GAPDH have been suggested. This is supported by the recently solved three-dimensional structure of two mammalian ALDHs (16, 17), in which differences regarding NAD⁺ binding, topology of the catalytic domain, and subunit association clearly indicate a functionally convergent evolution of phosphorylating and nonphosphorylating GAPDH.

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† The abbreviations used are: GAPDH, glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.12/E.C. 1.2.1.13); ALDH, aldehyde dehydrogenase; 3-PGA, 3-phosphoglycerate; GAP, glyceraldehyde 3-phosphate; GAPN, nonphosphorylating GAPDH; PCR, polymerase chain reaction.

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enzymes. Here, we describe functional, structural, and regulatory properties of the NAD\textsuperscript{+}-dependent nonphosphorylating GAPDH of \textit{T. tenax} and discuss them in terms of physiological and phylogenetic aspects.

**EXPERIMENTAL PROCEDURES**

### Chemicals and Plasmids—\textit{d}-GAP and \textit{n}-GAP were prepared from monobromide salts of the diethyl acetal (Sigma); all other chemicals (pro analysis grade) were from Fluka or Merck. Solutions of 1,3-bisphosphoglycerate were prepared as described previously (8). Cloning of PCR products and restriction fragments was performed using the plasmids pGEM \textit{T} (Promega) and pBluescipt \textit{II} K (Stratagene), respectively. For heterologous expression, the vector pJF118 EH via two new restriction sites (\textit{GEEVKAAVDRLRLAELDLRKIGGDYI}, peptide 1, \textit{RAGLLEGVIKEKGGVPVYPSTS}; peptide 2, \textit{NAGKPKSAAVGEV}-termined (peptide 2 overlaps with peptide 3 above position 30): peptide sequencing was performed by automated Edman degradation in a gas membranes (Applied Biosystems) by semidry electrotransfer (23). Isoelectric focusing system (Pharmacia) according to the manufacturer's protocol and with the recombinant protein (MRAGL, see above).

**Phylogenetic Analysis—** For sequence analysis and computer alignments, the programs GENMON, version 4.4 (GBF Braunschweig), and CLUSTAL W (28) were used. Homology searches were performed with BLASTP and BLASTX via MEDLINE. The source of sequence information was GenBank (update, June 1997). Phylogenetic trees were calculated with the PHYLIP program package, version 3.5c (29), and reliability of branches was estimated by bootstrap analyses. The PAUP program, version 3.1, was used for maximum parsimony analysis of protein sequences, including bootstrap replicates.

**RESULTS**

### Nucleotide Sequence of the Gene Coding for NAD\textsuperscript{+}-dependent GAPDH from \textit{T. tenax}—The sequence analysis revealed a single open reading frame comprising 1503 base pairs (Fig. 1, positions 130–1632) corresponding to a polypeptide of 501 amino acid residues with a calculated molecular mass of 55 kDa. The deduced amino acid sequence corresponds with the partial amino acid sequences of the three \textit{CNBr} fragments prepared from the protein and a tryptic peptide published earlier (8), thus identifying the open reading frame as the coding gene for the NAD\textsuperscript{+}-dependent GAPDH. The translation start could not be determined by Edman degradation, presumably due to N-terminal modification. Translation is probably initiated at the AUG codon of positions 130–132, where the encoded methionyl residue represents the N-terminal cleavage site of \textit{CNBr} peptide 1, because in the upstream region no potential start codon was found up to the next in-frame stop codon at position 106–108. In front of the coding region, a sequence resembling the boxA element of archaical promoters was identified, suggesting functional importance as transcriptional signal. A putative ribosome binding site is located at positions 119–124, matching the complementary 3′-end of the 185 rRNA of \textit{T. tenax} (30).

### Deduced Amino Acid Sequence of the NAD\textsuperscript{+}-dependent GAPDH: Comparison with Homologous Proteins—The NAD\textsuperscript{+}-dependent GAPDH shows significant sequence similarity with aldehyde dehydrogenases of various sources but not with phosphorylating GAPDH from Bacteria, Eucarya, or Archaea. The close relationship of the \textit{T. tenax} enzyme to ALDH is reflected by accordance in active site residues assigned on the basis of the recently resolved three-dimensional structure of rat liver ALDH3 (Fig. 2) and bovine ALDH2 (16, 17). The comparison shows that in addition to the catalytically essential Glu-209 (263) and Cys-243 (297), all residues involved in NAD\textsuperscript{+} binding are strictly conserved in the \textit{T. tenax} sequence: Asn-114 (168), Thr-186 (242), Gly-187 (243), Leu-210 (264), Gly-211 (265), and Phe-395 (397) (numbering according to the ALDH3 sequence; numbers in parentheses refer to \textit{T. tenax}) (see Fig. 2). The highest overall similarity was detected to GAP-specific ALDH from pea and mungo (31.1 and 31.4\% amino acid identity, respectively) and to the bacterium \textit{S. mutans} (33.5\% identity; Fig. 2), characterized as GAPN.

**Phylogenetic Analyses—** Phylogenetic analyses using parsimony and distance matrix (neighbor-joining) methods were performed with various representatives of the ALDH superfamily (Fig. 3), including the predicted protein sequences of
genes from the genomes of Methanococcus jannaschii (31), Synechocystis sp. (32), and Rhizobium meliloti (33). The analyses resulted in a complex tree topology similar to that found by Habenicht et al. (14), which is characterized by several poorly resolved lineages comprising enzymes of different substrate specificities (Fig. 3). The NAD$^+$-GAPDH of T. tenax is affiliated with the GAPN from plants and S. mutans. Although this branch is not robustly supported by bootstrap analysis, the affiliation of T. tenax GAPDH with the GAPN-subtree is assured by several unique sequence signatures (Fig. 2) (GEW (26–28), EEV (55–57), PFNYP (106–170), IVLEDADL (271–278), and GQRC (294–297) (numbers in parentheses are the positions of the T. tenax sequence).

Expression of the Gene Encoding NAD$^+$-dependent GAPDH in E. coli and Comparison of the Recombinant Protein with the Enzyme Isolated from T. tenax Cells—For functional and structural studies, the gene encoding NAD$^+$-dependent GAPDH (gapN) was expressed in E. coli. As calculated from the specific activity of the purified enzyme (Table I and Fig. 4), the expression efficiency in E. coli was relatively low (1% of the total soluble protein was recombinant GAPDH). Comparisons between the GAPDH isolated from T. tenax and the recombinant enzyme revealed no differences with respect to molecular mass and enzymic properties, such as kinetic parameters of cosubstrate saturation in the presence and absence of the effector AMP (Table I).

With respect to thermal stability, the recombinant enzyme differed from the enzyme isolated from T. tenax cells. Although both enzymes showed the same initial inactivation rates at 100 °C (pseudo-first order kinetics: $t_{1/2}$ = 52 min up to 30 min of incubation), the inactivation rates differed with preceding incubation time: the activity of the enzyme isolated from T. tenax cells decreased less dramatically, resulting in a residual activity of 30% after 100 min of incubation compared with 10% for the recombinant enzyme. Possibly, the modification responsible for the N-terminal block of the protein from T. tenax influences its thermal stability.

Reaction Catalyzed by the Enzyme—Contrary to previous results (8), enzyme preparations from T. tenax cells did not show activity in the reverse reaction, neither with 1,3-bisphospho-
phosphoglycerate nor with 3-PGA as substrate (range of 1,3-bisphosphoglycerate or 3-PGA, 0.5–10 mM), strongly suggesting that the enzyme works exclusively in the oxidative direction like all other ALDHs known at present. The irreversibility of the reaction could also be confirmed with the recombinant enzyme. Possibly, impurities in the previous enzyme preparations mimicked a reversible reaction. For analyzing further enzymic properties of the enzyme, such as substrate or cosubstrate specificity, and the effect of various metabolites on the enzyme activity, we exclusively used the functionally equivalent but more convenient recombinant enzyme.

The enzyme proved to be specific for D-GAP. L-GAP acts as strong competitive inhibitor with respect to D-GAP ($K_i = 130 \text{ mM}$). As a consequence, saturation kinetics with a racemic mixture of d-isomer and l-isomer revealed a 50% lower $V_{max}$. The saturation with d-GAP followed classical Michaelis-Menten kinetics, showing half-maximal saturation at 50 $\mu$M. A definite $K_m$ for the free aldehyde, the presumed substrate of the enzyme, cannot be given because the portion of the free aldehyde in aqueous solution could not be determined at 70 °C. None of the following aldehydes and alcohols (concentration range, 0.5–20 mM) tested for their ability to act as substrates (assay without substrate) and to compete for the active site (assay in the presence of half-saturating substrate concentration) were accepted by the enzyme: formaldehyde, acetaldehyde, propionaldehyde, $n$-valeraldehyde, butyraldehyde, benzaldehyde, hexanal, glyceraldehyde, glycolic aldehyde, succinic semialdehyde, and betaine aldehyde. The enzyme uses exclusively NAD$^+$ as a cosubstrate ($K_m$ of NAD$^+$ is 3.0 mM). NADP$^+$ cannot replace NAD$^+$ but acts as strong inhibitor (see below).

### Effectors of the NAD$^+$-dependent GAPDH

Several metabolites, including NADP$^+$, NADPH, NADH, and the adenine nucleotides ATP, ADP, and AMP, were tested under nonsaturating substrate and cosubstrate concentrations (0.2 mM d-GAP; 1 mM NAD$^+$) as possible effectors for the enzyme. Virtually no effects were observed with dihydroxyacetone phosphate, phosphoenol pyruvate, coenzyme A, erythrose 4-phosphate, xylonate, dihydroxyacetone phosphate, and glucose 6-phosphate. Figure 2 shows the amino acid sequence alignment of the NAD$^+$-dependent GAPDH from *T. tenax* with various ALDHs.

![Amino acid sequence alignment of the NAD$^+$-dependent GAPDH from *T. tenax* with various ALDHs.](image)

Gaps introduced for optimal alignment are indicated by hyphens. Conserved functional residues are in boldface and unique sequence signatures of nonphosphorylating GAPDHs are shaded. Amino acid positions and secondary structure elements (α, α-helix; β, β-strand) of the ALDH3 from rat liver (17) are given above the sequences. Origin of sequences: DHAP $\Rightarrow$ RAT, nonphosphorylating GAPDH of *T. tenax* (this study); GDN STRMU, nonphosphorylating GAPDH of *Pseudomonas sativum* (14); GAPN MAIZE, nonphosphorylating GAPDH of *Zea mays* (14); ALDH $\Rightarrow$ M. jannaschi (31); ALDH RHIME, unspecified ALDH from *M. jannaschi* (31); ALDH PHOR, unspecified ALDH from *Photorhabdus luminescens* (32); ALDH $\Rightarrow$ R. meliloti (33).

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**Fig. 2.** Amino acid sequence alignment of the NAD$^+$-dependent GAPDH from *T. tenax* with various ALDHs. Gaps introduced for optimal alignment are indicated by hyphens. Conserved functional residues are in boldface and unique sequence signatures of nonphosphorylating GAPDHs are shaded. Amino acid positions and secondary structure elements (α, α-helix; β, β-strand) of the ALDH3 from rat liver (17) are given above the sequences. Origin of sequences: DHAP $\Rightarrow$ RAT, nonphosphorylating GAPDH of *T. tenax* (this study); GDN STRMU, nonphosphorylating GAPDH of *Pseudomonas sativum* (14); GAPN MAIZE, nonphosphorylating GAPDH of *Zea mays* (14); ALDH $\Rightarrow$ M. jannaschi (31); ALDH RHIME, unspecified ALDH from *M. jannaschi* (31); ALDH PHOR, unspecified ALDH from *Photorhabdus luminescens* (32); ALDH $\Rightarrow$ R. meliloti (33).
5-phosphate, fructose 1,6-phosphate, or sedoheptulose 7-phosphate, whereas NADP⁺, NADPH, NADH, and ATP acted as potent inhibitors (apparent \(K_D\) 0.3–3000 \(\mu\)M, as calculated from their concentration-dependent inhibition) (Table II). ADP, AMP, glucose 1-phosphate, glucose 6-phosphate, fructose 1-phosphate, fructose 6-phosphate, and ribose 5-phosphate acted as activators (apparent \(K_D\) 1.0–2500 \(\mu\)M, as calculated from their concentration-dependent activation) (Table II). NADPH and glucose 1-phosphate proved to be the most affine effectors, exhibiting apparent \(K_D\) values of 0.3 \(\mu\)M and 1.0 \(\mu\)M, respectively, under the conditions applied. In the presence of both NADPH and glucose 1-phosphate at equivalent concentrations (10 \(\mu\)M), the effect of the activator predominated, resulting in a 2-fold higher activity as compared with the control without effector. The compensating effect of glucose 1-phosphate on the inhibitory action of NADPH was also reflected by an approximately 200-fold lowering of the apparent \(K_D\) of NADPH in the presence of 10 \(\mu\)M glucose 1-phosphate (apparent \(K_D\) of NADPH \(= 56 \mu\)M; data not shown). Mg²⁺ ions did not affect the enzymic properties, either alone or in combination with adenine nucleotides.

The effects of NADP⁺, NADPH, ATP, ADP, AMP, and glucose 1-phosphate on the activity of the enzyme were studied in more detail by investigating the influence on NAD⁺ and D-GAP binding at saturating concentrations of the nonvaried substrate. All ligands affect exclusively the affinity of the enzyme for NAD⁺.
**TABLE I**

| NAD⁺-dependent GAPDH of T. tenax | Isolated from T. tenax | Isolated from E. coli |
|----------------------------------|------------------------|-----------------------|
| **NAD⁺ saturation**             |                        |                       |
| Without AMP                      |                        |                       |
| $V_{max}$ (units/mg)             | 36.5                   | 38.0                  |
| $K_{m}$ (mM)                     | 3.3                    | 3.1                   |
| In the presence of AMP           |                        |                       |
| $V_{max}$ (units/mg)             | 37.0                   | 37.5                  |
| $K_{m}$ (mM)                     | 1.4                    | 1.5                   |
| **Arsenate saturation**         |                        |                       |
| $V_{max}$ (units/mg)             | 37.0                   | 39.0                  |
| Apparent $K_{m}$ (mM)            | 75.0                   | 75.0                  |
| **Thermal stability**            |                        |                       |
| Residual activity (%) after 100 min at 100 °C | 30 | 10 |

**Molecular mass**

| Subunit (kDa) | Native (kDa) |
|---------------|--------------|
| 55,000        | 220,000      |

**Phylogenetic tree**

**Fig. 4.** Electropherogram of SDS-polyacrylamide gel electrophoresis documenting the purification of the NAD⁺-dependent GAPDH from T. tenax and from E. coli cells. M, molecular mass standard; CE, crude extract; HP, fraction after heat precipitation; QS, fraction after separation on Q-Sepharose; HA, fraction after chromatography on hydroxylapatite; BS, fractions after chromatography on blue Sepharose.

Additionally, most of the effectors induce positive cooperativity of cosubstrate binding (Table III and Fig. 5), with maximal Hill coefficients of 1.9 as exhibited by NADH.

**DISCUSSION**

NAD⁺-dependent GAPDH of T. tenax, a Member of the ALDH Superfamily—Reaction type and sequence features classify the NAD⁺-dependent GAPDH of T. tenax as ALDH. As such, the enzyme represents the first biochemically characterized archaean member of this highly diverse protein family, which comprises a variety of enzymes differing in their enzymic properties, including substrate and cosubstrate specificity and mode of catalysis (14, 34). Regarding the high substrate specificity for D-GAP and the phosphate independence of the catalyzed oxidation of the aldehyde to the corresponding acid, 3-PGA, the T. tenax enzyme resembles most the ALDH subgroup of NADP⁺-specific GAPN. Differences from presently known GAPN exist with respect to cosubstrate specificity and regulatory properties.

**TABLE II**

| Metabolite tested | Apparent $K_{d}$ (μM) |
|-------------------|------------------------|
| Inhibitors        |                        |
| NADPH             | 0.3                    |
| NADP⁺             | 1.0                    |
| NADH              | 30                     |
| AMP               | 3000                   |
| Activators        |                        |
| Glucose 1-phosphate | 1.0            |
| AMP               | 140                    |
| Fructose 6-phosphate | 200              |
| ADP               | 270                    |
| Fructose 1-phosphate | 1700             |
| Ribose 5-phosphate | 2500               |

**Effect of various metabolites on cosubstrate binding to the NAD⁺-dependent GAPDH of T. tenax**

**Fig. 5.** Cosubstrate saturation of NAD⁺-dependent GAPDH of T. tenax in the presence of various effectors. Assay conditions: 90 mM HEPES (pH 7.0), 160 mM KCl, 4 mM DL-GAP. ●, control; ○, 50 μM NADP⁺; ■, 100 μM glucose 1-phosphate; □, 1 mM AMP.

**Phylogeny**—Despite rather low bootstrap support (45%, neighbor joining), the affiliation of the T. tenax enzyme with the GAPN lineage is revealed by unique sequence signatures (Fig. 2). Two of these sequences (GQRC and PFNYP) could be assigned to the active site region on the basis of crystallographic studies of mammalian ALDH (16, 17), emphasizing their importance for evolutionary affinity. The first fragment harbors the catalytically essential cysteinyl residue (corresponding to Cys-234 in the rat liver ALDH3), and the second contains a functionally important asparaginyl residue (corresponding to Asn-114 in the rat liver ALDH3) interacting with the nicotinamide ring of the cosubstrate. The functional importance of a third signature sequence (IVLEDADL) is still speculative. On the basis of the three-dimensional structure of the mammalian ALDH, it is part of the loop region between β₁ and
α4 helix of a second α/β dinucleotide binding fold constituting most of the catalytic domain. Because this loop interacts commonly with the phosphate moiety of nucleotides in nucleotide-binding proteins, it is conceivable that this region assumed the function of effector binding in GAPN.

Because the monophyletic GAPN subtree comprises members of all three domains (it is presumed that the relationship between GAPN enzymes is not confounded by lateral gene transfer events), it appears that the GAPN lineage originated prior to the divergence of the domains.

As shown in Fig. 3, the root of the GAPN subtree implied by the deeper branching lineages, leading to the uncharacterized ALDHs of *R. meliloti*, *M. jannaschii*, or *S. hygroscopicus*, indicates a closer affinity between eucaryal and bacterial GAPN under the exclusion of the archael homolog of *T. tenax*. As such, the branching order does not coincide with the "conventional" topology of rooted universal trees constructed with elongation factors, aminoacyl-tRNA synthetases, and ATPases (36–38), showing the archael and eucaryal homologs as sister groups and the bacterial counterparts as earliest diverging line. Thus, with the presently available GAPN homologues a universal-tree topology could be verified, which may reflect the bacterial inheritance of the eucaryal genome. This topology is also supported by the preferred similarity between the bacterial and eucaryal homologs of several metabolic enzymes, such as phosphorylating GAPDH, 3-phosphoglycerate kinase, and triosephosphate isomerase (39–43).

The other, apparently more deeply rooting branches in Fig. 3 bear both bacterial and eucaryal sequences. They may witness an intense gene diversification already in the progenote, as recently suggested (14). Alternatively, because no archaeal members within these lineages could be identified yet, they may reflect bacterial ALDH radiation, whereby the interlacing of bacterial and eucaryal ALDHs could be due to the bacterial origin of eucaryal ALDH genes.

Allosteric Regulation—One of the most striking features of the *T. tenax* enzyme is its regulation by metabolites, which is not only more pronounced than that of all GAPNs but also of all ALDHs characterized to date. As a main difference from eucaryal and bacterial GAPN, the NAD⁺-dependent GAPDH of *T. tenax* is not only inhibited but also activated by a series of metabolites. Until now, stimulating effects could only be described for the *S. mutans* GAPN (13) with cations such as NH₄⁺ and K⁺. But this activation is rather due to decreasing substrate inhibition and probably not of physiological relevance. Generally, little is known about the regulation capacity of other ALDHs with broad or narrow substrate specificity. Activation by Mg²⁺ ions has been reported for the mitochondrial ALDH from horse liver or rat testis (44, 45). Furthermore stimulation of the esterolytic activity of ALDH by NAD⁺ or NADH has been described with a more pronounced effect of pyridine nucleotides (and also coenzyme A) observed in the case of methylmalonate semialdehyde dehydrogenase (34). Still, it is an open question whether the hydrolytic activity of ALDH is of physiological importance.

At present, our structural and functional information is too scarce to deduce a consistent model for the effector-induced substrate or cosubstrate binding of the *T. tenax* enzyme. At least, Hill coefficients of (maximally) 2, determined for the NAD⁺ binding of the *T. tenax* enzyme in the presence of effectors, are consistent with the subunit arrangement of the mammalian ALDH expecting cooperativity between the two closely neighbored cosubstrate binding sites.

No information is available about the binding site(s) of the different effectors or about the conformational changes caused by them. On the basis of the known three-dimensional structure of ALDH, one may speculate that effector binding of the nonphosphorylating GAPDH occurs at the catalytic domain. In the ALDH structure model, the subunit contains two α/β dinucleotide binding folds; the first (functional) fold is located in the NAD⁺ binding domain, and the second (obviously functionless) fold is found in the catalytic domain. The suggestion that in GAPN, including the *T. tenax* enzyme, the second dinucleotide binding unit may be involved in effector binding is based mainly on the finding that in these enzymes a specific sequence conservation could be observed concerning a region that corresponds to the loop region connecting β₁ and α₁ of that second dinucleotide binding unit. The close vicinity of the presumed effector site to the NAD⁺ binding pocket and to the dimer intersubunit contacts would explain the effector-mediated influence on both NAD⁺ binding affinity and cooperativity.

Physiological Role—From the pattern of regulation, conclusions about the physiological role of the *T. tenax* enzyme can be drawn. The observation that the activity of the enzyme is controlled mainly by the energy charge of the cell and by intermediates of glucan polymer degradation (glucose 1-phosphate) and glycolysis (fructose 6-phosphate) accounts for a catabolic role of the enzyme, which thus differs from eucaryal or bacterial GAPN with obvious anabolic function (10, 13).

*T. tenax* possesses two catabolic pathways for glucose, the modified (nonphosphorylative) Entner-Doudoroff pathway and a variant of the Embden-Meyerhof-Parnas pathway representing the dominant catabolic route in cells grown on glucose (6, 7, 46). From its reaction type and regulation pattern, the NAD⁺-dependent nonphosphorylating GAPDH fits into the catabolic reaction sequence of the Embden-Meyerhof-Parnas pathway of this organism.

Previous suggestions based on the strong inhibition of the enzyme by NAD⁺ suggested an in vivo activity only at late stationary phase, when the intracellular concentration of the inhibitor is sufficiently low. But in fact, the enzyme also seems to be active under normal growth conditions because the enzyme exhibits activity despite strong inhibition by NAD⁺ and NADPH as soon as activators are simultaneously present. Glucose 1-phosphate proved to be the most potent activator. It represents the first intermediate in the degradation of glyco- gen, a reserve polymer that has been previously documented in *T. tenax* (47). As reported here, 10 μM glucose 1-phosphate is sufficient to reduce the affinity of the enzyme for the strongest inhibitor NADPH by a factor of 200. From the in vitro experiments, we must therefore assume a tight in vivo regulation of the enzyme, allowing activity only at a low ATP/ADP+AMP ratio and/or in the presence of activating intermediates, such as glucose 1-phosphate and fructose 6-phosphate. Whether the activation by fructose 1-phosphate and ribose 5-phosphate is of physiological relevance, exhibiting a significantly lower affinity for the enzyme, remains to be established.

With its regulatory properties, NAD⁺-dependent GAPDH fulfills the control function commonly executed by the enzyme couple ATP-dependent phosphofructokinase/fructose-1,6-bisphosphatase in glycolysis, which is substituted by the reversible nonregulated PP₁-phosphofructokinase in *T. tenax*. Furthermore, the irreversible reaction catalyzed by the NAD⁺-dependent GAPDH of *T. tenax* drives the carbon flux into the catabolic direction of the pathway. Thus, the enzyme seems to compensate not only the lacking regulatory properties of the reversible PP₁-phosphofructokinase but also its driving force for the catabolic reaction. This ability is gained at the expense of 2 mol of ATP/mol of glucose, which would be additionally generated using a phosphorylating but reversible GAPDH. Thus, the principal physiological function of the NAD⁺-dependent GAPDH should reside in an increase of the catabolic rate and the recovery of compounds such as 3-PGA, phosphoenolpyruvate, or...
pyruvate, allowing a more rapid availability of ATP and/or precursors for biosynthetic purposes. In terms of a high glycolytic energy yield, however, the use of the phosphorylating NADP-dependent GAPDH should be more effective. Therefore, the possibility cannot be excluded that under conditions of high energy demand this enzyme is active in catabolism as well, despite a higher \( V_{\text{max}} \) for the reductive reaction, suggesting an anabolic role (8).

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