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FEBS Letters 339 (1994) 97–100

FEBS 13644

FEBS LETTERS

Arguments against a close relationship between non-phosphorylating and phosphorylating glyceraldehyde-3-phosphate dehydrogenases

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Received 15 November 1993; revised version received 31 December 1993

Abstract

Non-phosphorylating NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (EC 1.2.1.9) from spinach leaves was purified to homogeneity using an improved purification procedure. Thus, a major contaminant with molecular mass and ion-exchange properties similar to non-phosphorylating GAPDH was eliminated. Using this pure non-phosphorylating GAPDH, cofactor stereospecificity was determined by ^{1}H NMR. Analysis of the NADPH formed from the hydride transfer from glyceraldehyde-3-phosphate to [4-^{2}H]NADP showed that the enzyme belongs to the A-stereospecific dehydrogenase family. This stereospecificity is the same as that described for the aldehyde dehydrogenase (ALDH) superfamily and opposite to that of the phosphorylating GAPDH. Moreover, results from peptide sequencing analysis suggest a similarity in sequence between the non-phosphorylating GAPDH and ALDHs. Thus, the results taken all together strongly suggest that non-phosphorylating GAPDH belongs to the ALDH family and has no close relationship to the phosphorylating GAPDH class.

Key words: Stereospecificity; NMR; Glyceraldehyde-3-phosphate dehydrogenase; Spinach; Evolution

1. Introduction

Glyceraldehyde-3-phosphate dehydrogenases (GAPDH) exist as two classes of enzymes which can be either phosphorylating or non-phosphorylating. The cytosolic non-phosphorylating GAPDH (EC 1.2.1.9) which is present only in photosynthetic eukaryotic organisms ([1] and references cited therein) and certain non-photosynthetic prokaryotes [2], catalyzes the irreversible oxidation of glyceraldehyde-3-phosphate (G3P) into 3-phosphoglycerate (3PG). It was proposed that this enzyme plays a shuttle role in transferring photosynthetic reducing power from the plant chloroplast stroma to the cytoplasm [3]. The structure and the chemical mechanism of action of this enzyme are not well known. However it has been established that this strictly NADP- dependent enzyme is composed of 4 subunits of about 50 kDa each [4], most probably possesses a cysteine residue essential for its activity [5] and operates according to a steady-state random reaction mechanism [6,7]. The phosphorylating GAPDH catalyzes the reversible oxidation of G3P into 1,3-diphosphoglycerate (1,3 dPG). Two different types are known which are both composed of four subunits of about 36 kDa each: (i) a strictly NAD- dependent enzyme (EC 1.2.1.12) , which is involved in the glycolytic cycle and the gluconeogenesis in the cytoplasm of most organisms [8]; (ii) a chloroplastic enzyme (EC 1.2.1.13) , which participates in photosynthetic CO_2 fixation and which exhibits a dual-coenzyme specificity with a preference for NADP [9,10], although the NADP-dependent activity is strongly regulated and often low [11,12]. More is known about the structure and the chemical mechanism of phosphorylating GAPDHs. In particular, high resolution three-dimensional structures of the homotetrameric glycolytic GAPDH from *Bacillus stearothermophilus* [13] and *Escherichia coli* (Dideberg, personal communication) are now available and the mechanism of the catalytic process has been the subject of numerous studies using either kinetic analysis ([14] and references cited therein) or site-directed mutagenesis [15,16]. In particular, the currently accepted forward reaction pathway involves two steps: first, an oxidoreduction which leads to the formation of an acylenzyme intermediate via an essential cysteine residue and NADH; and second, a phosphorylation which is favored by the presence of NAD and produces 1,3 dPG [8].

Recently, using site-directed mutagenesis, the essential nucleophile Cys 149 in the NAD-dependent phospho-

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Abbreviations: B. stearothermophilus, Bacillus stearothermophilus; E. coli, Escherichia coli; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ALDH, aldehyde dehydrogenase; 1,3 dPG, 1,3-diphosphoglycerate; G3P glyceraldehyde-3-phosphate; 3PG, 3-phosphoglycerate; PTH-amino acids, phenylthiohydantoin amino acids.

SSDI 0014-5793(94)00028-T

rylating GAPDH from *E. coli* was changed to Ala. The resulting mutant exhibited a non-phosphorylating dehydrogenase activity [16]. Moreover, various replacements of amino acids which belong to the coenzyme subsite of GAPDH from *B. stearothermophilus* [17,18] and from *E. coli* (Clermont et al., unpublished results) conferred activity with NADP. Thus, it is possible by protein engineering to convert in vitro a NAD-phosphorylating GAPDH into an efficient NADP-non-phosphorylating GAPDH.

This finding raises questions about a possible evolutionary relationship between the NAD(P)-dependent phosphorylating GAPDHs and the NADP-dependent non-phosphorylating GAPDH. To address this problem, various approaches can be used including comparison of protein sequences, three-dimensional structures or the nature of the chemical mechanisms of both enzymes. Unfortunately, very little is known so far about the non-phosphorylating GAPDH. Hence, we used an approach which relies on determining the stereospecificity of cofactor reduction in order to probe the possible relationship between the non-phosphorylating and the phosphorylating GAPDH. Indeed, Garavito et al. [19] suggested that all evolutionary related dehydrogenases retained the original coenzyme stereospecificity. Furthermore, peptide sequencing analyses are also reported.

2. Materials and methods

2.1. Materials

NADP was obtained from Boehringer. The enzymes (yeast aldehyde dehydrogenase and L-glutamate dehydrogenase from bovine liver) as well as all the enzyme substrates were purchased from Sigma. Chromatographic media were from Pharmacia except for hydroxyapatite (Bio-Gel HTP) which was obtained from Bio-Rad.

2.2. Purification of the non-phosphorylating NADP-dependent GAPDH from spinach leaves

The initial steps of the purification were as described by Scagliarini et al. [6] except for the addition of 0.5 mM phenylmethylsulfonyl fluoride to buffer A (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 14 mM β-mercaptoethanol). Following acetone fractionation the preparation was desalted by a Sephadex G-25 (2.5 × 50 cm) previously equilibrated with buffer B (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 7 mM β-mercaptoethanol) and the enzyme was purified using a FPLC system (Pharmacia, Uppsala) with a Q-Sepharose column (1.6 × 10 cm) equilibrated with buffer B and thermostated at 10°C. The eluent used was buffer B (30 ml), followed by a linear gradient (0–0.3 M KCl) in buffer B at a flow rate of 2 ml · min^{-1}. The non-phosphorylating GAPDH was eluted at around 170 mM KCl. Pooled fractions were kept overnight at −20°C with addition of 0.2 mM NADP and 10% (V/V) glycerol

Q-Sepharose-purified fractions were concentrated by ultrafiltration (Amicon 52 cell, PM 10 membrane) and equilibrated with buffer C (10 mM Na-phosphate, pH 7.0) in a Sephadex G-25 column. The enzyme was then charged on a hydroxyapatite column (1.6 × 4.5 cm) equilibrated with buffer C at 10°C. After washing it with 25 ml buffer C, a two-step gradient was applied, using 25 ml each of 200 mM and then 250 mM Na-phosphate (pH 7.0). The non-phosphorylating GAPDH is eluted with 250 mM Na-phosphate. At this step its specific activity is of the order of 100–140 μmol · min^{-1} · mg^{-1} (Table I). An occasional 100 kDa contaminant detected by SDS-PAGE can be removed by passage of the enzyme previously equilibrated in 10 mM Tris-HCl, pH 7.5, 0.2 mM EDTA and 2 mM β-mercaptoethanol through a Blue-Sepharose CL 6 B column (1 × 10 cm) at room temperature. The Blue-Sepharose does not retain the non-phosphorylating GAPDH while it adsorbs the contaminant.

Protein concentration was estimated by a modified Lowry method [20] using bovine serum albumine as standard and in the final stages of purification by absorbance at 280 nm using an extinction coefficient of 1.0 mg^{-1} · ml · cm^{-1}.

Assays of the non-phosphorylating GAPDH were performed as previously described by Scagliarini et al. [6].

2.3. Preparation of [^{4}A-^{2}H]NADPH

Aldehyde dehydrogenase (an A-stereospecific dehydrogenase) was used to prepare [^{4}A-^{2}H]NADPH with deuterated acetaldehyde, as previously described by Levy and Betts [21]. The reaction was stopped by ultrafiltration through an Amicon-30 membrane and the [^{4}A-^{2}H]-NADPH was purified by chromatography on an anion exchange Q Sepharose column (FPLC Pharmacia) using an elution gradient of ammonium bicarbonate (0–1 M) at pH 9.5. The corresponding fractions were pooled and lyophilised. Complete deuteration at the C-4 position was checked by ^{1}H NMR in D_2O with sodium trimethysilyl propionate (TSP) as an internal standard (Table 2).

2.4. Preparation of the [4-^{2}H]NADP

Preparation of the [4-^{2}H]NADP was performed by incubating of [4A-^{2}H]NADPH (1 mM), 2-ketoglutarate (2 mM) and ammonia (120 mM) in 25 mM sodium phosphate buffer at pH 7.3 in the presence of 20 units of L-glutamate dehydrogenase, a B-stereospecific enzyme [22]. The reaction was performed at 26°C until the equilibrium was reached. The enzyme was removed by ultrafiltration and [4-^{2}H]NADP was purified and collected as previously described for the purification of [4A-^{2}H]NADPH. Complete deuteration at the C-4 position was checked by ^{1}H NMR.

2.5 Determination of the stereospecificity of the non-phosphorylating GAPDH

The non-phosphorylating GAPDH reaction mixture (25 ml) contained 0.5 mM [4-^{2}H]NADP, 1 mM G3P, 25 mM triethanolamine buffer, 2 mM EDTA and 10 mM β-mercaptoethanol. The pH of the reaction mixture was adjusted to 8.2. The reaction was started by adding 5 units of the non-phosphorylating GAPDH and incubated at 26°C for 5 h. The reaction was stopped by removing enzyme through ultrafiltration. The NADPH thus obtained was purified as previously described and then analyzed by ^{1}H NMR.

2.6. Microsequencing of peptides from non-phosphorylating GAPDH

Peptide sequences were determined as previously described by

Table 1
Purification scheme of the non-phosphorylating NADP-dependent GAPDH from 1 kg spinach leaves

Steps	Activity (μmol · min^{-1})	Protein (mg)	Specific activity (μmol · min^{-1} · mg^{-1})	Purification (fold)	Yield (%)
Homogenate	280	6214	0.045	1	100
$(NH_4)_2SO_4$	221	635	0.335	7.7	79
Acetone	214	127	1.68	37	76
Q-Sepharose	122	10.5	11.6	258	44
Hydroxyapatite	48.2	0.461	104	2313	17
Blue Sepharose	37.5	0.305	123	2732	13

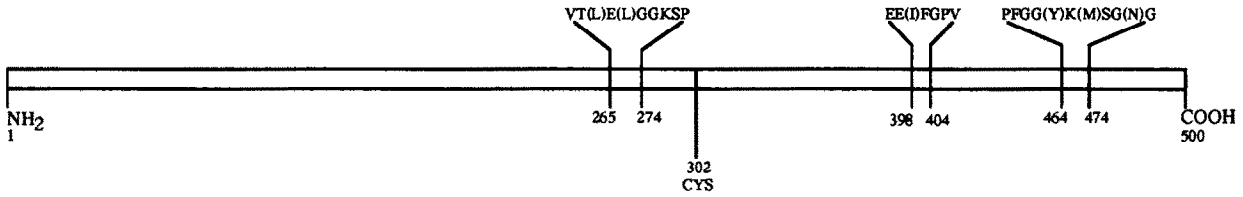

Fig. 1. Schematic representation of non-phosphorylating ALDHs. A total of 22 mammalian, fungal, plant and bacterial ALDHs with known primary structures from the Swiss-Prot and Gen EMBL databases have been taken into account. The numbering of the sequence is according to the horse cytoplasmic enzyme [28]. Sequence alignment was performed with the program PILEUP from the GCG program suite [29] which employs a method similar to that of Hogeweg and Hesper [30]. The three almost conserved regions present in all non-phosphorylating ALDH are shown. ( ) indicates not strictly conserved amino acids. Cys-302 is postulated to be involved in the formation of the thioacylenzyme intermediate [31].

Dequard-Chablat et al. [23], except that the tryptic digestion of the non-phosphorylating GAPDH was performed in 100 mM phosphate buffer, pH 8 containing 4 M urea.

3. Results and discussion

A pure enzyme preparation is required for the determination of cofactor stereospecificity as well for amino acid sequence analysis of the non-phosphorylating GAPDH. For that purpose, an improved purification method has been set up. The hydroxyapatite step permitted to eliminate a major contaminant which co-migrates with non-phosphorylating GAPDH in SDS-PAGE. Accordingly, the isolated non-phosphorylating GAPDH exhibits an up to 10-fold increase of specific activity (Table 1) when compared to enzyme preparations obtained using previously described methods [4–6].

Determination of the cofactor stereospecificity of the non-phosphorylating GAPDH was performed using [4-^{2}H]NADP. The NADPH formed during the non-reversible conversion of G3P into 3PG shows a ^{1}H NMR signal at 2.84 ppm which corresponds to the C4-A pyridinium hydrogen (Table 2). This clearly demonstrates that the hydride transfer from aldehydic substrate occurs at the A-side of the nicotinamidinium ring. Non-phosphorylating GAPDH is therefore an A-stereospecific dehydrogenase. This stereospecificity appears to be the opposite of that found for the glycolytic and the chloroplastic GAPDHs [21,24] while it is the same as that of classical aldehyde dehydrogenases (ALDH) [24,25]. This suggests that the non-phosphorylating GAPDH belongs to the aldehyde dehydrogenase superfamily. ALDHs constitute a large family of related enzymes that catalyze the irreversible oxidation of a wide variety of aldehydes to their corresponding acids via the formation of a thioacyl intermediate [26]. From the protein sequence alignment, three regions of nearly complete identity can be identified between all known non-phosphorylating ALDHs (Fig. 1). Peptide sequencing analyses of the spinach non-phosphorylating GAPDH were recently untertaken with the aim of cloning the corresponding gene. The sequences of the four isolated peptides are shown in Table 3. 7 residues (shown by bold letters, see Table 3) of peptide 2, i.e. EEPFGPV, are almost identical, with just one conservative replacement, to one of the invariant ALDH regions (Fig. 1). By contrast this sequence is not present in phosphorylating GAPDHs, a class of enzymes which presents more than 40% of sequence identity [27]. Thus, our results taken all together strongly suggest that the non-

Table 2
Stereospecificity of hydride transfer

	Proton at C-8 and C-2 of adenine		Proton at C-2 of dihydro-nicotinamide	Proton at C-4 of dihydro-nicotinamide	
NADPH[a]	8.48	8.24	6.94	2.82	2.74
[4-^{2}H]NADPH[b]	8.46	8.23	6.92	2.70	
[4-^{2}H]NADPH[c]	8.46	8.24	6.92	2.84	

^{1}H NMR data of NADPH. Chemical shifts are taken from sodium trimethylsilyl [^{2}H]propionate internal standard (pH 7.6). NADPH concentration was 1.5 mM. The spectra were taken at 25°C with a Bruker AM 400 MHz spectrophotometer. The chemical shifts of protons at C-8 and C-2 of adenine ring and C-2 of the dihydronicotinamide ring are indicated as internal reference.
[a]Commercial NADPH; [b][4-^{2}H]NADPH obtained by reduction of NADP by yeast aldehyde dehydrogenase; [c]NADPH obtained by reduction of [4-^{2}H]NADP by the non-phosphorylating NADP-dependent GAPDH.

Table 3
Amino acid sequences of the non-phosphorylating GAPDH peptides generated by trypsine

Peptides	Sequences	Amounts of sequenced peptides
1	(S) TVINLPTPTYTM (M)	40 pmol
2	(I) (A) ? **EEPFGPV**LPVI (K)	25 pmol
3	(V) IPIFL (Y) (T) VNL (A) V	20 pmol
4	($^H/_E$) GNLI?PLLLDNV ($^N/_V$) PD ($^M/_D$)	25 pmol

Uncertain amino acids are shown in parentheses and not determined amino acids are indicated by '?'. Bold letters indicate the sequence corresponding to the second conserved region of aldehyde dehydrogenases (see Fig. 1). Peptides were obtained after tryptic digestion of about 1 nmol of non-phosphorylating GAPDH. Peptides were isolated to homogeneity by two successive purification steps [23] and purified peptides were analyzed by Edman degradation. Amounts of sequenced peptides are shown in the table.

phosphorylating GAPDH belongs to the aldehyde dehydrogenase family and argue against an ancestral relationship between the non-phosphorylating and the phosphorylating GAPDHs. This would support the hypothesis that the catalytical mechanism used by the non-phosphorylating GAPDH is similar to that described for ALDHs, which involves the formation of a thioacyl-enzyme intermediate, and thus excludes an alternative mechanism in which the true substrate is the gem-diol entity instead of the aldehyde form [16]. More generally, this study is consistent with the concept that similarities in properties, in the case of GAPDH both classes use the same substrate (G3P) and cofactor (NAD(P)) and presumably catalyze the oxidation of the substrate via the same chemical intermediate, is not a sufficient argument to conclude in favor of a close relationship between two classes of enzymes.

Acknowledgements: This work was supported by grants of the Centre National de la Recherche Scientifique, the Université de Nancy I, the Institut de Biotechnologie de Nancy, the Ministero dell'Universita e Ricerca and the Consiglio Nazionale delle Ricerche, Rome. The authors would like to acknowledge Dr C. Corbier for helpful advices and V. Pint for her excellent technical assistance.

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