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Purification and Characterization of Cytosolic Glyceraldehyde-3-phosphate Dehydrogenase from the Dromedary Camel

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Abstract Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (EC 1.2.1.12), a key enzyme of carbon metabolism, was purified and characterized to homogeneity from skeletal muscle of *Camelus dromedarius*. The protein was purified approximately 26.8 folds by conventional ammonium sulphate fractionation followed by Blue Sepharose CL-6B chromatography, and its physical and kinetic properties were investigated. The native protein is a homotetramer with an apparent molecular weight of approximately 146 kDa. Isoelectric focusing analysis showed the presence of only one GAPDH isoform with an isoelectric point of 7.2. The optimum pH of the purified enzyme was 7.8. Studies on the effect of temperature on enzyme activity revealed an optimal value of approximately 28–32 ºC with activation energy of 4.9 kcal/mol. The apparent K_m values for NAD^+ and DL-glyceraldehyde-3-phophate were estimated to be 0.025±0.040 mM and 0.21±0.08 mM, respectively. The V_{max} of the purified protein was estimated to be 52.7±5.9 U/mg. These kinetic parameter values were different from those described previously, reflecting protein differences between species.

Key words *Camelus dromedarius*; glyceraldehyde-3-phosphate dehydrogenase; protein purification; skeletal muscle

Dromedaries have an important socio-economic role in the arid regions and occupy arid regions of the Middle East through northern India and arid regions in Africa, most notably, the Sahara Desert. They have been introduced to arid regions of central Australia where some of the only feral populations now persist [1]. The original range of their wild ancestors was probably south Asia and the Arabian peninsula. *Camelus dromedarius* prefers desert conditions characterized by a long dry season and a short rainy season. Introduction of dromedary camels into other climates has proven unsuccessful because they are sensitive to cold and humidity [1]. *Camelus dromedarius* has the ability to survive under harsh environments in areas where other ruminants have failed. Indeed, dromedaries are able to benefit from vegetation generally rejected by other species [2] and can survive without food and water for several days [3]. Such behavior assumes an energy metabolism that can provide energy when food intake does not occur for long periods. It requires metabolic and respiratory changes.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an enzyme involved in the central pathways of carbon metabolism [4]. Three distinct GAPDH proteins have been found with different subcellular localizations, each performing various roles. The typical NAD^+-dependent glycolytic enzyme (EC 1.2.1.12) is found in all organisms so far studied and is located in the cytoplasm. It plays a pivotal role in the Embden-Meyerhoff pathway not only in glycolysis but also in glyconeogenesis [4]. The $NADP^+$-dependent GAPDH (EC 1.2.1.13), a key component of the reductive pentose-phosphate cycle, is located in the chloroplast stroma and the cyanobacterial cytoplasm and is involved in photosynthetic CO_2 assimilation [5,6]. These two enzymes catalyze the oxidation of glyceraldehyde-3-phosphate (G3P) into diphosphoglyceric acid using the

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orthophosphate anion (PO_4^{3-}) as a cofactor in their catalytic reactions. The cytosolic non-phosphorylating $NADP^+$-dependent GAPDH (EC 1.2.1.9), also named Pi-independent G3P: $NADP^+$-oxidoreductase, catalyzes an irreversible oxidation of G3P to 3-phosphoglycerate [5,7].

The glycolytic NAD^+-dependent GAPDH has been remarkably conserved during evolution, having a homotetrameric structure with subunits of 35–37 kDa [4]. GAPDH has been isolated from a variety of species [8], including mesophilic, moderately thermophilic and hyperthermophilic microorganisms [9]. These enzymes have been shown to be highly similar in amino acid sequence, subunit composition and enzymatic behavior [8]. Comparison of these homologs might shed light on the mechanism of adaptation to extreme environmental conditions at the molecular level. The skeletal muscle enzyme, composed of four identical subunits, has been used as a model to study the adaptation of some species to the environmental conditions [10,11].

In the present study, we have asked whether some of the distinguishing characteristics of other GAPDHs are in any way evident in the features of *C. dromedarius* GAPDH. The aim of this paper was to isolate and biochemically characterize the skeletal muscle GAPDH from *C. dromedarius*.

Materials and Methods

Materials

The muscle tissues of dromedaries (Settat, Morocco) were taken after deathettat, Morocco). The samples were immediately placed in liquid nitrogen. DL-glyceraldehyde-3-phosphate (DL-G3P) was prepared from monobarium salts of diethyl acetal (Sigma, St. Louis, USA). All other chemicals (analytical grade) were from Fluka (St. Gallen, Switzerland) or Merck (Darmstadt, Germany).

Enzyme purification

The enzyme was purified to electrophoretic homogeneity from crude cell extracts by the procedure previously described [10,17]. All steps were performed at 4 ºC. Centrifugations were carried out at 15,000 *g* for 45 min.

Preparation of crude extracts

Skeletal muscle tissue (approximately 9 g, fresh weight) was ground and homogenized using an Ultra-Turrax homogenizer T25 basic (Fisher Bioblock Scientific, Illkirch, France) in 25 mM Tris-HCl buffer (pH 7.5), containing 2 mM EDTA, 10 mM 2-mercaptoethanol and 2 mM phenylmethylsulfonyl fluoride (protease inhibitor) at a ratio of 5 ml/g fresh tissue. The supernatant (soluble protein fraction) obtained after centrifugation was considered as the crude extract.

Ammonium sulphate fractionation

The crude extract was subjected to protein precipitation in the 66%–88% (*W*/*V*) saturation range of ammonium sulphate. The final pellet was dissolved in a minimal volume of solution containing 25 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 10 mM 2-mercaptoethanol (buffer A). The protein solution was dialyzed twice against 2 liters of the same buffer.

Blue Sepharose CL-6B chromatography

The dialyzed enzyme preparation was applied to a Blue Sepharose CL-6B column (1 cm×6 cm) equilibrated with two bed volumes of buffer A.

The column was washed with three bed volumes of buffer A and two bed volumes of the same buffer adjusted to pH 8.5 (buffer B). The enzyme was eventually eluted with buffer B containing 10 mM NAD^+ at a flow rate of 6 ml/h. Active fractions were collected.

GAPDH activity assay and protein concentration

GAPDH activity in the oxidative phosphorylation was determined spectrophotometrically at 25 ºC by monitoring NADH generation at 340 nm [7]. The reaction mixture (1 ml) contained 50 mM Tricine-NaOH buffer (pH 8.5), 10 mM sodium arsenate, 1 mM NAD^+ and 2 mM DL-G3P. A coupled assay in which aldolase (1 U/ml) produced the stoichiometric breakage of *D*-fructose 1-6 bisphosphate (2 mM) to DL-G3P and dihydroxyacetone-phosphate, the first product being the actual substrate of the oxidative reaction [19], was usually used during enzyme purification. One unit of enzyme is defined as the amount which catalyses the formation of 1 mM of NADH per minute under the conditions used. Protein was estimated by the method of Bradford [20] using bovine serum albumin as a standard. Activity levels in cell-free extracts were expressed as specific activity.

Polyacrylamide gel electrophoresis (PAGE)

Determination of native molecular weight was carried out by electrophoresis on non-denaturing polyacrylamide slab gels (Bio-Rad, Hercules, USA) using the following protein standards ferritin (440 kDa), catalase (232 kDa), aldolase (154 kDa) and ovalbumin (43 kDa). As described by the method of Hedrick and Smith [14], a calibration

curve can be calculated from the relative mobilities of standard proteins on non-denaturing polyacrylamide gels with different acrylamide concentrations, 5%, 7%, 9%, and 10% (*W*/*V*).

Isoelectric focusing [21] was carried out with the same electrophoresis system in 5% polyacrylamide slab gels holding ampholite-generated pH gradients in the range 3.5–10.0 (pharmalite 3.5–10; Pharmacia Biotech, Uppsala, Sweden), 25 mM NaOH and 20 mM acetic acid as cathode and anode solutions, respectively. The isoelectric point (pI) protein markers kit used was the Bio-Rad IEF standards pI range 4.45–9.60 for isoelectric focusing.

Sodium dodecyl sulfate (SDS)-PAGE was performed as described by Laemmli [22] on one-dimensional 12% polyacrylamide slab gels containing 0.1% SDS. Gels were run on a miniature vertical slab gel unit (Hoefer Scientific Instruments, San Francisco, USA). After electrophoresis, gels were stained with 0.2% (*W*/*V*) Coomassie brilliant blue R-250 in methanol:acetic acid:water at a ratio of 4:1:5 (*V*/*V*/*V*) for 30 min at room temperature. The apparent subunit molecular weight was determined by measuring relative mobilities and comparing with the pre-stained SDS-PAGE molecular mass protein standards (Precision Plus Protein; Bio-Rad).

Preparation of polyclonal antibodies

Polyclonal antibodies were raised in New Zealand white rabbits to GAPDH that had been purified to electrophoretic homogeneity from dromedary skeletal muscle. The enzyme (approximately 0.3 mg) was mixed with Freund's complete adjuvant and injected subcutaneously into rabbits in multiple places as described by Vaitukaitis [23]. Rabbits were boosted every three weeks and bleeding was done after 10 days.

Western blot analysis

Proteins were separated by SDS-PAGE as described previously. Separated protein bands were electrophoretically transferred from the gel slab to a nitrocellulose filter (Schleicher & Schuell, Keene, USA) using a Bio-Rad Trans-Blot system. Transferred proteins were then visualized by pre-staining in 0.2% (*W*/*V*) ponceau red in trichloroacetic acid. The nitrocellulose paper was then incubated for 1 h in blocking solution containing 5% (*W*/*V*) non-fat dry milk, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.01% (*W*/*V*) NaN_3 and 0.05% (*V*/*V*) Tween-20, followed by incubation with the anti-GAPDH antiserum (1:1000 dilution) as the first antibody. Western blots were eventually visualized by coupled immunoreaction with a peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody (1:1000; Boehringer Mannheim, Hamburg, Germany) as the second antibody using 4-chloro-1-naphtol as a chromogenic substrate.

Kinetic studies of purified GAPDH

For kinetic studies, DL-G3P in aqueous solution was used as described above at a final concentration of 2 mM. Kinetic constants were calculated from initial rates estimated from initial absorbance changes. To determine the kinetic parameters, the concentration of the respective fixed substrate for the reaction was 1 mM NAD^+ or 0.2 mM G3P in the presence of 10 mM PO_4^{3-}. K_m and V_{max} were determined from Lineweaver-Burk double reciprocal plots.

Influence of pH and temperature on the purified GAPDH

To determine optimal pH, enzymatic activity was measured over a wide range of pH (from 4 to 10) with different buffers (acetate, imidazole, Tris and carbonate-bicarbonate) adjusted to the same ionic strength as that of the standard reaction mixture. To determine the optimal temperature, reactions were carried out in the 20–80 ºC temperature range using a thermostated cuvette holder connected with a refrigerated bath circulator.

Effects of cations on GAPDH activity

The purified *C. dromedarius* GAPDH was incubated in the presence of various concentrations of cations. For each concentration, residual activity was measured at different times of incubation in comparison with the control (without cation). The I_{50} values were calculated using a Job plot [24], in which data can be plotted as V_0/V_i versus the concentration of an inhibitor. After plotting data for several different cation concentrations, a straight line is drawn through the points and the cation concentration that corresponds to V_0/V_i=2 is the I_{50} value.

Results and Discussion

Purification of *C. dromedarius* GAPDH

GAPDH was purified from a soluble protein fraction from dromedary skeletal muscle to electrophoretic homogeneity using a procedure involving conventional dye-affinity chromatography, as previously reported for other NAD^+-dependent GAPDHs [10,12,13]. Chromatography on Blue Sepharose is a very effective and straightforward purification, therefore no additional purification steps were

Table 1 Purification of glyceraldehyde-3-phosphate dehydrogenase from skeletal muscle of *Camelus dromedarius*

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Purification (fold)	Yield (%)
Crude extract	385.0	646.8	1.68	1.0	100
Ammonium sulphate [a]	25.0	397.5	15.90	9.5	61
Blue Sepharose CL-6B	3.4	153.3	45.10	26.8	23

[a] ammonium sulphate 66%–88%.

required to obtain homogeneous preparations of the enzyme.

Table 1 summarizes a representative purification protocol. Values of approximately 45.1 U/mg of protein were obtained for the purified enzyme with a yield of approximately 23% and a purification factor of approximately 26.8 folds.

SDS-PAGE analysis of the different fractions obtained during the purification procedure showed a progressive enrichment in a 37 kDa protein [**Fig. 1(A)**]. Only this protein band, having the same size as the GAPDH subunit, was seen in the electrophoretically homogeneous final enzyme preparations [**Fig. 1(A)**, lane 3].

Polyclonal antibody against purified dromedary GAPDH was used for Western blot analysis. This antibody clearly recognized a purified protein and a single protein band of 37 kDa, corresponding to the GAPDH subunit, in crude extracts from skeletal muscle [**Fig. 1(B)**].

Physicochemical and kinetic properties of purified GAPDH from dromedary

The molecular mass of the native enzyme was performed using different separating gels in the absence of SDS according to the Hedrick and Smith method [14]. The molecular mass of the native GAPDH was estimated to be 146 kDa (**Fig. 2**). SDS-PAGE of the purified GAPDH showed, as stated above, a single stained band corresponding to a 37 kDa protein (**Fig. 1**, lane 3), indicating that *C. dromedarius* GAPDH should have a homotetrameric structure like other GAPDHs [4].

Isoelectric focusing of the protein separation according to pI values showed a single protein band at pI 7.2 (the estimated pI for the enzyme) (**Fig. 3**). This result indi-

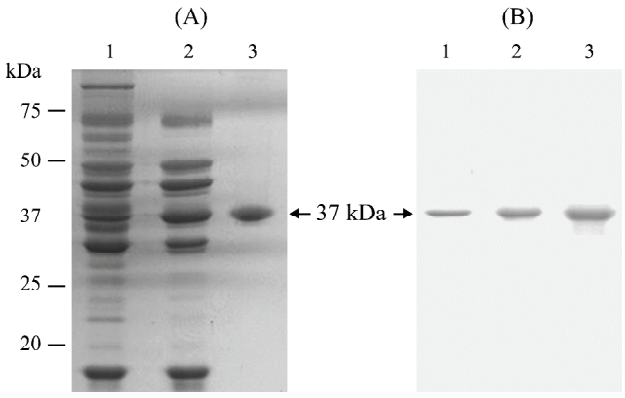

Fig. 1 Purification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from skeletal muscle of *Camelus dromedarius*
(A) Coomassie brilliant blue-stained sodium dodecylsulfate-polyacrylamide gel electrophoresis electrophotogram showing different purification step fractions. 1, cell-free protein extracts; 2, 66%–88% ammonium sulphate protein fraction; 3, Blue Sepharose fraction pool (pure protein preparation). (B) Western blot analysis using a monospecific antibody against the purified GAPDH. 1, cell-free protein extracts; 2, 66%–88% ammonium sulphate protein fraction; 3, Blue Sepharose fraction pool. Aliquots of cell extracts (approximately 25 μg of protein per lane) were used. The arrow points to the 37 kDa GAPDH subunit band.

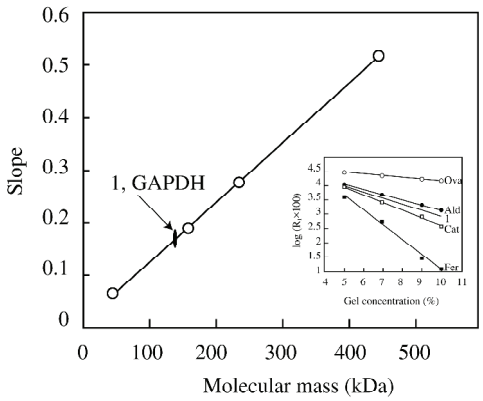

Fig. 2 Determination of native dromedary glyceraldehyde-3-phosphate dehydrogenase (GAPDH) molecular weight by non-denaturing polyacrylamide gel electrophoresis
Proteins were electrophoresed on various acrylamide concentration gels (concentration range 5%, 7%, 9% and 10%) under non-denaturing conditions. Molecular weight marker proteins were ferritin (Fer, 440 kDa), catalase (Cat, 232 kDa), aldolase (Ald, 154 kDa) and ovalbumin (Ova, 43 kDa). Relative mobilities of proteins plotted as log R versus acrylamide concentration are indicated on the inset. A plot of the obtained slopes versus molecular weight was linear and used to determine native dromedary GAPDH molecular weight.

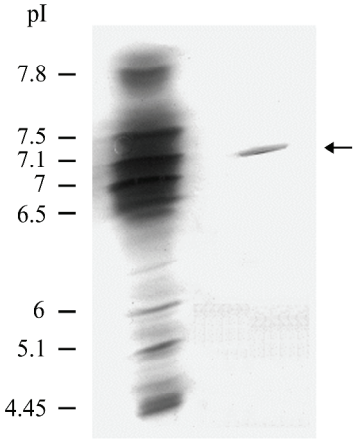

Fig. 3 Isoelectric focusing in polyacrylamide slab gel (5%, *W*/*V*, acrylamide) holding an ampholyte-generated pH gradient (pH range, 3.5–10) of purified dromedary glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Aliquots containing approximately 20 μg protein were applied per lane. The arrow points to the GAPDH isoelectric point (pI) of 7.2.

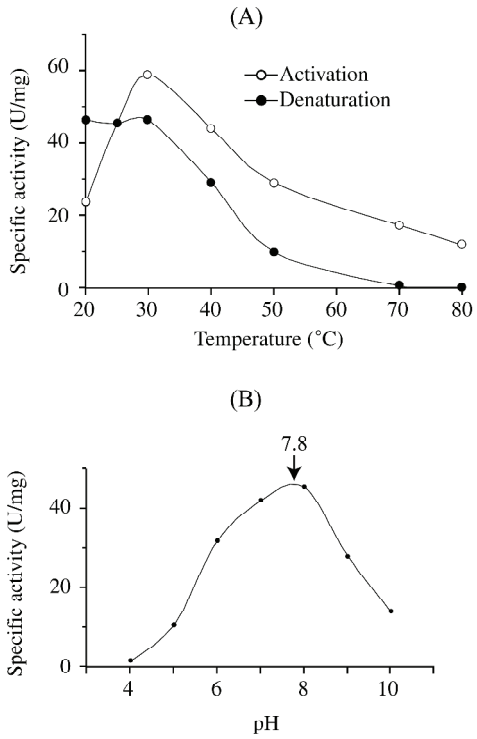

Fig. 4 Influence of temperature and pH level on enzymatic activity of purified dromedary glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

(A) Enzymatic activity of purified GAPDH from skeletal muscle of *Camelus dromedarius* at a temperature range from 20 ºC to 80 ºC, showing the effects of the assay temperature (activation) and the enzyme pre-incubating temperature (denaturation). (B) Enzymatic activity of purified GAPDH in the pH of 4.0–10.0 using a mixture of different buffers. Values are given as means of three separate experiments.

cates that only one slightly basic isoform of the enzyme occurs in skeletal muscle of the dromedary, and strongly suggests that a single GapC gene is expressed in this tissue. A single GAPDH isoform has also been found in some other animal tissues and microorganisms, both prokaryotes and eukaryotes [15], but it seems this is not the general rule as the presence of several GAPDH isoforms has been reported in organisms [12,16,17].

Pre-incubation of dromedary GAPDH for 10 min at temperatures varying between 20 and 32 ºC did not irreversibly affect the enzyme activity. Thermal inactivation, however, occurred above 35 ºC and resulted in total activity loss at 70 ºC [**Fig. 4(A)**]. Studies on the effect of temperature on enzyme activity revealed an optimal value of approximately 28–32 ºC with activation energy 4.9 kcal/mol calculated using the corresponding linear Arrhenius plot (data not shown). The optimal pH value for the oxidative reaction was 7.8 [**Fig. 4(B)**] with considerable activity in the range 7–8.5.

As GAPDH catalyzes a two-substrate reaction, the K_m values for NAD^+ and G3P were determined from double-reciprocal plots of reaction rates under standard conditions in the presence of saturating concentrations of one substrate (**Table 2**). Apparent K_m values for NAD^+ and DL-G3P were estimated to be 0.025±0.01 mM and 0.21±0.08 mM, respectively. The V_{max} of the purified protein was estimated to be 52.7±5.9 U/mg. Although the K_m for G3P is similar to those found for cytosolic GAPDHs from jerboa liver and human tissues [17,18], the K_m for NAD^+ of the *C. dromedarius* GADPH is clearly lower.

Table 2 Kinetic constants (K_m and V_{max}) for oxidation reaction of *Camelus dromedarius* glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

	K_m (DL-G3P) (mM)	K_m (NAD^+) (mM)	V_{max} (U/mg)
Cd GAPDH	0.21±0.08	0.025±0.010	52.7±5.9

Cd GAPDH, *Camelus dromedarius* GAPDH.

Influence of cations on GAPDH activity

To determine the effect of metal ions on the activity of *C. dromedarius* GAPDH, the purified enzyme was incubated *in vitro* in the presence of reagents. All experiments were performed at the same enzyme

concentration. The results are reported in **Table 3**. The cations listed were all inhibitors but the degree of inhibition depended upon the nature of the added ion. Thus, the activity of purified GAPDH was strongly inhibited by Cd^{2+} and Hg^{2+} with an I_{50} value of 4.6±0.5 mM and 6.2±0.9 mM, respectively. The K^+ showed a weaker inhibition with an I_{50} value of 34.6±1.9 mM.

Table 3 Effect of cations on *Camelus dromedarius* purified glyceraldehyde-3-phosphate dehydrogenase activity

Cation	I_{50} (mM)
Cd^{2+}	4.6±0.5
Hg^{2+}	6.2±0.9
Zn^{2+}	14.2±1.1
Cu^{2+}	22.7±1.2
Mn^{2+}	25.8±1.3
K^+	34.6±1.9

This inhibition could be explained several ways and the following hypotheses can be put forward: (1) excess ions interact with some amino acid residues in the active center of GAPDH and in this way diminish the binding of a substrate; and/or (2) these ions bind to the amino acid residues in the active center but cause a partial conformational change of the GAPDH molecule altering its catalytic properties, which results in a decrease in enzymatic activity.

Conclusion

We purified to homogeneity and characterized dromedary GAPDH with a molecular mass of 37 kDa. The kinetic parameter values differed in a number of instances from those described previously, reflecting protein differences between species [17,18]. In addition, we sequenced the gene corresponding to this protein to establish the phylogenetic relationships of the dromedary enzyme, the first GAPDH from a camelid studied so far, with other GAPDHs of eukaryotic and prokaryotic cells.

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