Eur Biophys J (2008) 37:1139–1144
DOI 10.1007/s00249-008-0332-x

ORIGINAL PAPER

Adjustment of conformational flexibility of glyceraldehyde-3-phosphate dehydrogenase as a means of thermal adaptation and allosteric regulation

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Received: 28 September 2007 / Revised: 9 April 2008 / Accepted: 11 April 2008 / Published online: 1 May 2008

Abstract Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Thermotoga maritima* (TmGAPDH) is a thermostable enzyme (T_m = 102°C), which is fully active at temperatures near 80°C but has very low activity at room temperature. In search for an explanation of this behavior, we measured the conformational flexibility of the protein by hydrogen–deuterium exchange and compared the results with those obtained with GAPDH from rabbit muscle (RmGAPDH). At room temperature, the conformational flexibility of TmGAPDH is much less than that of RmGAPDH, but increases with increasing temperature and becomes comparable to that of RmGAPDH near the physiological temperature of *Thermotoga maritima*. Using the available three-dimensional structures of the two enzymes, we compared the B factors that reflect the local mobility of protein atoms. The largest differences in B factors are seen in the coenzyme and NAD binding regions. The likely reason for the low activity of TmGAPDH at room temperature is that the motions required for enzyme functions are restricted. The findings support the idea of "corresponding states" which claims that over the time span of evolution, the overall conformational flexibility of proteins has been preserved at their corresponding physiological temperatures.

Regional Biophysics Conference of the National Biophysical Societies of Austria, Croatia, Hungary, Italy, Serbia, and Slovenia.

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Keywords Conformational flexibility · Thermal adaptation · Protein stability · Glyceraldehyde-3-phosphate dehydrogenase · Allosteric regulation

Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) catalyzes the sixth step of glycolysis and thus serves to break down glucose for energy and carbon. In addition to this long established metabolic function, GAPDH has recently been implicated in several non-metabolic processes including transcription activation (Zheng et al. 2003), initiation of apoptosis (Hara et al. 2005), and ER to Golgi vesicle shuttling (Tisdale and Artalejo 2007).

GAPDH is a tetramer of identical subunits, each comprising a NAD-binding and a catalytic domain (Bolotina et al. 1966; Dalziel et al. 1981). NAD binding results in a conformational change (Leslie and Wonacott 1984) and subsequent binding events occur with decreasing affinity, resulting in negative cooperativity between the four subunits (Conway and Koshland 1968). NAD binding also results in a decrease of conformational flexibility (Zavodszky et al. 1966). Using hydrogen/deuterium (H/D) exchange monitored by electrospray mass spectrometry, Williams and coworkers demonstrated that the dynamics of the protein reduces upon the binding of each NAD molecule (Williams et al. 2006). The reduction is largest when the first NAD molecule binds; smaller reduction occurs upon the binding of the second and third NAD molecules, and no change in dynamics is observed upon the binding of the fourth NAD molecule (Williams et al. 2006).

GAPDH has been isolated from a number of organisms, including an extremely thermostable variant from the

eubacterium *Thermotoga maritima* (Wrba et al. 1990). The X-ray structure of *T. maritima* GAPDH (TmGAPDH) was determined (Korndörfer et al. 1995), and, in accord with the results of homology modeling (Szilágyi and Závodszky 1995), a large number of salt bridges was identified as an important factor contributing to the high thermostability of the protein.

The activation energy of the oxidation of glyceraldehyde-3-phosphate by TmGAPDH was found to decrease with increasing temperature, and H/D exchange measurements showed that TmGAPDH is significantly more rigid at room temperature than GAPDH isolated from yeast (Wrba et al. 1990). The authors concluded that GAPDH supports the hypothesis of "corresponding states" (Jaenicke and Závodszky 1990), which claims that over the timespan of evolution, the overall conformational flexibility of proteins from mesophiles and thermophiles has been preserved at their corresponding physiological temperatures. A prime example supporting this concept is isopropylmalate dehydrogenase (IPMDH): it was shown that while IPMDH from *Thermus thermophilus* is significantly more rigid at room temperature than IPMDH from *Escherichia coli*, their flexibilities are nearly equal at the physiological temperatures of these two organisms (Závodszky et al. 1998). However, examples of proteins from thermophilic organisms with unexpectedly high flexibility have been found (Hernandez et al. 2000), and it has become obvious that the "rigidity hypothesis" must not be taken dogmatically (Jaenicke 2000).

Although TmGAPDH was found to be of limited flexibility at room temperature, and near-UV circular dichroism and fluorescence spectra indicated a tight packing of aromatic side chains in their hydrophobic environment (Wrba et al. 1990), the temperature dependence of fluorescence spectra revealed an anomalous behavior. Proteins commonly show a red shift of their fluorescence emission when temperature is increased, due to an increase in the mobility and accessibility of buried aromatic residues. But in the case of TmGAPDH, the opposite effect is observed: increasing temperature leads to a small blue shift accompanied by a small change in fluorescence intensity, indicating restricted flexibility of the aromatic side chains and further tightening of the hydrophobic interactions (Wrba et al. 1990).

This anomalous behavior of TmGAPDH may suggest that, in accordance with the tightening of hydrophobic interactions, the conformational flexibility of the enzyme might not increase as expected with increasing temperature, and thus the concept of "corresponding states" might not apply. Because the conformational flexibility of TmGAPDH has not yet been measured directly at the physiological temperature, we performed this measurement using H/D exchange. For comparative measurements, we used rabbit muscle GAPDH (RmGAPDH) as a mesophilic counterpart to TmGAPDH. The X-ray structure of RmGAPDH has recently been determined (Cowan-Jacob et al. 2003), which allows us to interpret our results in terms of the known three-dimensional structure. In addition to flexibility measurements, we also investigate the relationship between enzyme function and flexibility for these two enzymes.

Materials and methods

Chemicals

DEAE-cellulose (DE-52) and phenyl-Sepharose were purchased from Whatman and Amersham Biosciences, respectively. For affinity chromatography, CH-Sepharose 4B-N6-(2-aminoethyl)-NAD was used; the material was kindly provided by A. F. Bückmann (GBF, Braunschweig). NAD^+, NADH, ATP, glyceraldehyde-3-phosphate (barium salt), 3-phosphoglyceric acid, yeast phosphoglycerate kinase, RmGAPDH were purchased from Boehringer; cystein hydrochlorid was from Sigma. All other chemicals were analytical grade substances from Merck. Milli Q ionexchanged waters was used for every measurement.

Cultivation of the *T. maritima* cells and isolation of TmGAPDH

Cells were grown as described by Huber et al. (1986) using a 300-l fermentor at 80°C under N_2 atmosphere. Isolation and purification of the *T. maritima* enzyme was carried out as described earlier (Wrba et al. 1990).

Enzyme assay

Enzyme activity was monitored by tracing the absorbance at 366 nm in a Jasco V-550 spectrophotometer equipped with a Grant thermostat using 1-cm thermostated glass cuvettes. Measurements were performed at a temperature range of 5–75°C for TmGAPDH, and 3–40°C for RmGAPDH. The measurements were conducted as described by Wrba et al. (1990).

DSC measurements

Calorimetric measurements were performed on DASM-4 (Institute of Protein Research, Russian Academy of Sciences, Pushchino), and VP-DSC (Microcal Inc, Northampton, MA) scanning microcalorimeters. The protein concentration was adjusted to 0.2–2 mg/ml and the heating rate was kept constant 1°C/min. The samples were dialyzed against the buffers used in the activity measurements, and

the dialysis buffer was used as a reference. Heat capacities were calculated as outlined by Privalov (1979).

H/D exchange

The kinetics of the H/D exchange in D_2O were measured by FT-IR spectroscopy on a Bruker IFS-28 FT-IR photometer using the procedure described earlier (Závodszky et al. 1998). The temperature was measured with a sensor attached directly to the CaF_2 cell windows. Measurements were carried out at 25 ± 0.1°C for the mesophilic and 25 ± 0.1°C and 68 ± 0.1°C for the thermophilic enzymes. The samples were dialyzed in Teorell–Stenhagen buffer solutions, pH 6.0 and 7.0, and were lyophilized above liquid nitrogen for 6 h. The loss of activity was negligible upon lyophilization. Aliquots of buffers were also lyophilized and used for background measurements.

Lyophilized samples (1 mg of protein) were dissolved in D_2O. The time of the addition of D_2O was taken as the start of the exchange. A series of IR-spectra (in the 400–4,000 cm^{-1} region) was recorded, starting at 30–40 s after initiating the H/D exchange. A spectral resolution of 2 cm^{-1} was used. The number of scans used for recording (i.e., the time of recording a spectrum) was adjusted to the speed of the exchange (4 scan measurements at the beginning and 128 scans towards the end).

H/D exchange spectra were interpreted assuming the EX_2 mechanism, and the kinetic data of the exchange were plotted as the ratio of the unexchanged protons, X, versus $\log(k_0\ t)$ in the form of relaxation spectra as described in detail in (Závodszky et al. 1975, 1981; Venyaminov et al. 1976). k_0 is the chemical exchange rate constant for solvent-exposed amide-groups.

Results and discussion

TmGAPDH is fully active near its physiological temperature (80°C) but its activity is very low at room temperature (Wrba et al. 1990). Comparing this behavior with that of RmGAPDH, we find that the activity of the latter also drops when temperature is reduced to around 0°C. The Arrhenius plots of both enzymes (Fig. 1) are nonlinear, indicating a drop in activation energy above a certain temperature. The two Arrhenius plots are similar in shape but shifted relative to each other by about 20°C. Convex Arrhenius plots are common for multistep enzymatic reactions (Wrba et al. 1990). Truhlar and Kohen (2001) have given a detailed analysis of this phenomenon in terms of elementary rate constants. The convex temperature dependent change in the reaction rate constant of GAPDH can be interpreted as a consequence of the change in the pattern of functionally relevant conformational fluctuations (Demchenko 1997).

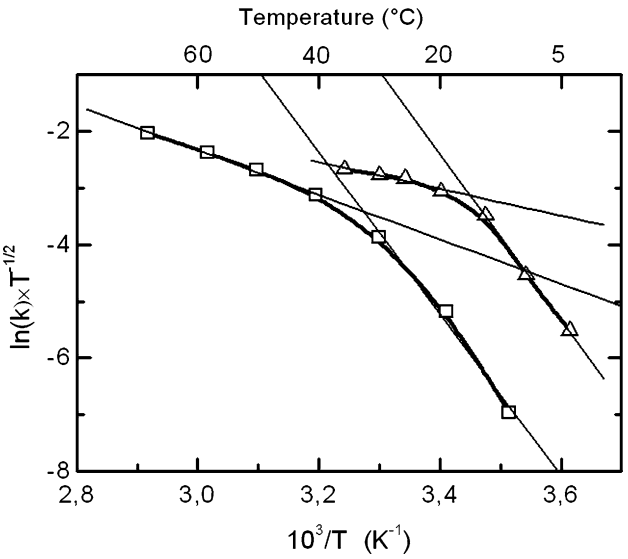

Fig. 1 Temperature dependence of the catalytic constant in the form of Arrhenius plots for the oxidation of glyceraldehyde-3-phosphate by TmGAPDH (*square*) and RmGAPDH (*triangle*). The two plots are shifted by about 20°C relative to each other, and both indicate a drop in activation energy above a certain temperature

What could be the reason for the drop in enzyme activity when temperature is decreased well below the physiological temperature? Wrba et al. (1990) suggested that a conformational change in the case of TmGAPDH may explain the temperature dependence of the activation energy. However, CD and fluorescence spectra only indicate a minor change. We measured the heat capacity curves of TmGAPDH and RmGAPDH by differential scanning microcalorimetry (Fig. 2). Apart from the peaks at the

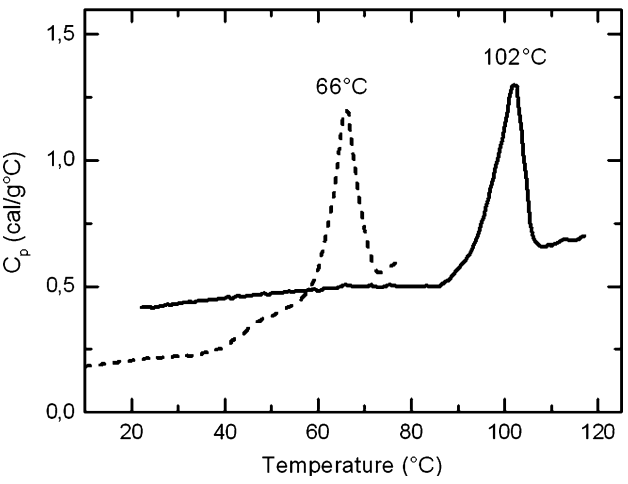

Fig. 2 Thermal denaturation of TmGAPDH (*solid line*) and RmGAPDH (*dashed line*) followed by differential scanning calorimetry. Measurements were performed with a constant heating rate of 1°C/min under the same conditions as for the enzyme activity assay. Melting temperatures are 66°C for RmGAPDH and 102°C for TmGAPDH

respective melting points (66 and 102°C for RmGAPDH and TmGAPDH, respectively), no deviations from the baselines are found, which suggests that no major conformational change occurs before approaching the melting point. Earlier, it was also demonstrated that TmGAPDH is not affected by cold denaturation or subunit dissociation at low temperature (Rehaber and Jaenicke 1992). If conformation is unchanged and the enzyme structure is intact at low temperatures, it is reasonable to assume that the drop in enzyme activity may be related to the conformational dynamics of the protein. For a number of enzymes, it has been convincingly demonstrated that conformational motion plays a vital role in enzyme activity (Hammes-Schiffer 2002). The fact that the activity of TmGAPDH at low temperature increases up to threefold upon addition of a small amount of guanidinium chloride (Rehaber and Jaenicke 1992) also suggests that it is the high rigidity of the enzyme that limits activity at low temperatures.

We measured the global conformational flexibility of both TmGAPDH and RmGAPDH, both at room temperature and at their respective physiological temperatures (25 and 68°C for RmGAPDH and TmGAPDH, respectively), by H/D exchange monitored by Fourier transform infrared spectroscopy. The H/D exchange relaxation curves are shown in Fig. 3. In accordance with the results of Wrba et al. (1990), TmGAPDH is found to be significantly more rigid at room temperature than its mesophilic counterpart (Fig. 3a). When TmGAPDH is measured at 68°C, however, its flexibility is found to be very close to that of RmGAPDH (Fig. 3b).

Thus, in spite of the tightening of aromatic-hydrophobic interactions indicated by changes in the fluorescence spectra (Wrba et al. 1990), the global flexibility of TmGAPDH still increases upon raising the temperature, and it approaches that of the mesophilic GAPDH. Thus, the behavior of GAPDH supports the idea of 'corresponding states' (Jaenicke and Závodszky 1990).

To see which regions differ most in flexibility between RmGAPDH and TmGAPDH, we compared the crystallographic temperature factors (B factors) found in the available X-ray structures of these proteins (PDB entries 1j0x and 1hdg, respectively). B factors reflect the local mobility of protein atoms, and were first used by Vihinen to compare the flexibility of mesophilic and thermophilic enzymes (Vihinen 1987). B factors usually depend on crystal contacts, resolution and the details of the refinement procedure (Carugo and Argos 1999), and therefore are generally not comparable between different structures without some normalization. However, the X-ray structures of RmGAPDH and TmGAPDH are very similar (the root mean square deviation of C_α positions is 1.31 Å), and their resolutions are very close (2.4 vs. 2.5 Å), therefore we chose to compare the absolute B factors to obtain a rough guide to identify the regions where flexibility differs most between the two structures.

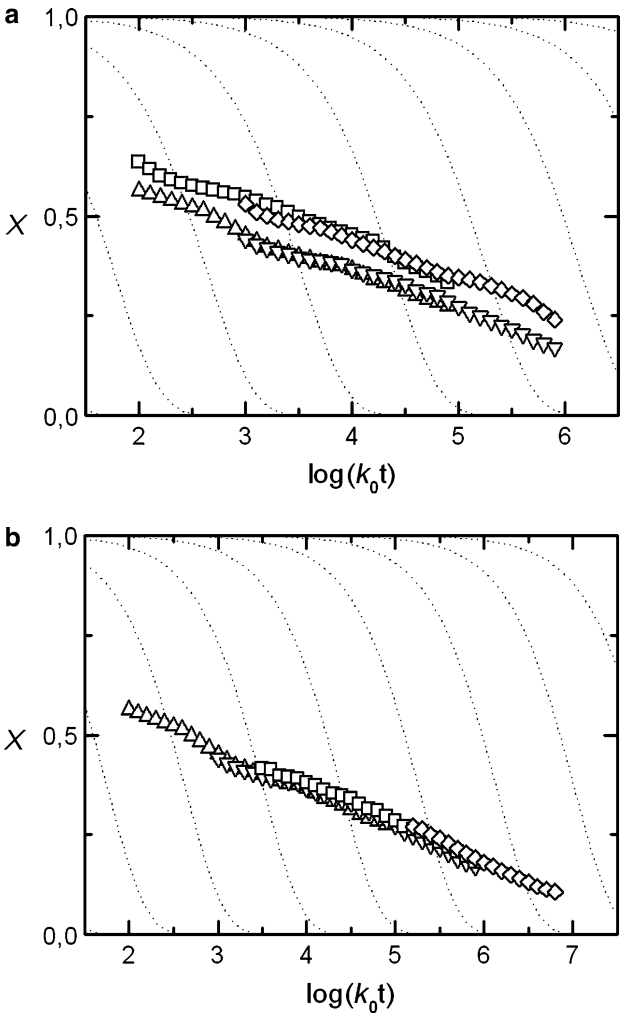

Fig. 3 **a** Hydrogen–deuterium exchange data summarized in the form of relaxation spectra for RmGAPDH (*up-triangles* pH 6; *down-triangles* pH 7) and TmGAPDH (*squares* pH 6, *diamonds* pH 7), measured at 25°C. *X* is the fraction of unexchanged peptide hydrogens, *t* is time, and k_0 is the chemical exchange rate constant. The *dotted lines* represent the exchange rate curves for hypothetical polypeptides characterized by constant probabilities of solvent exposure of the peptide groups. The data indicate a more rigid structure for the thermophilic enzyme. **b** Hydrogen–deuterium exchange data for TmGAPDH and RmGAPDH at two different pH values near their respective temperature optima (25°C for RmGAPDH and 68°C for TmGAPDH). The data show very similar flexibilities for the two enzymes

Figure 4 highlights those regions in the tetrameric structure where RmGAPDH is significantly more flexible than TmGAPDH. Most of the difference is observed in the NAD binding domain, and the largest differences are seen at the NAD binding region and at the substrate binding region. This indicates that flexibility adjustment is most prononunced in the regions that are vital for function.

These findings are in accord with the observations indicating reduction in the conformational dynamics when

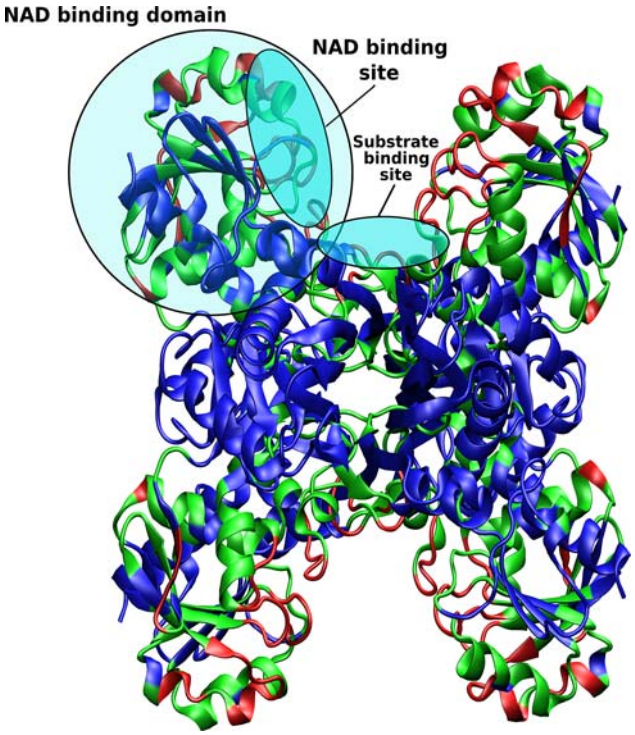

Fig. 4 The three-dimensional structure of RmGAPDH (PDB entry 1j0x) in its tetrameric form. The molecule was colored by the difference in C_α B-factors between RmGAPDH and TmGAPDH (PDB entry 1hdg), $\Delta B = B_{RmGAPDH} - B_{TmGAPDH}$. *Blue* $\Delta B < 9$; *green* $9 < \Delta B < 20$; *red* $\Delta B > 20$. Regions showing the highest difference in flexibility are colored *red*; these include the NAD binding and substrate binding sites, highlighted in one of the subunits

NAD molecules are bound (Williams et al. 2006; Zavodszky et al. 1966). GAPDH is an allosterically regulated enzyme, with the four subunits communicating with each other (Lakatos and Závodszky 1976). The structural comparison of apo- and holo-GAPDHs shows that, with the catalytic domains being in the same position, the NAD binding domain is rotated by 4.3° in the holo (NAD bound form), and there is a rearrangement of several residues involved in NAD binding (Leslie and Wonacott 1984). But the comparison of static structures does not provide the full picture about how allosteric signal transduction occurs; the data suggest that the fine-tuning of protein dynamics is essential for this process. Taken together, these observations indicate that dynamics in GAPDH is essential for both catalytic function and allosteric regulation, and that the flexibility of the protein, especially in the regions important for function, is adjusted to the relevant physiological temperature, maximizing the effectiveness of the enzyme near the optimum growth temperature of the organism.

Acknowledgments Péter Závodszky was supported by the Hungarian National Science Foundation (OTKA) Grants T046412, and NI-61915. A.S. was supported by a Bolyai János fellowship.

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