Crystal structure of two ternary complexes of phosphorylating Glyceraldehyde-3-Phosphate Dehydrogenase from *Bacillus stearothermophilus* with NAD and D-Glyceraldehyde-3-Phosphate.

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Running title: Structure of GAPDH in complex with NAD and substrate.

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Summary

The crystal structure of the phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Bacillus stearothermophilus* was solved in complex with its cofactor (NAD) and its physiological substrate, D-Glyceraldehyde-3-phosphate (D-G3P). In order to isolate a stable ternary complex, the nucleophilic residue of the active site, C149, was substituted with alanine or serine. The C149A and C149S GAPDH ternary complexes were obtained by soaking the crystals of the corresponding binary complexes (enzyme/NAD) in a solution containing G3P. The structures of the two binary and the two ternary complexes are presented. The D-G3P adopts the same conformation in the two ternary complexes. It is bound in a non-covalent way, in the free-aldehyde form, its C3-phosphate group being positioned in the Ps site and not in the Pi site. Its C1 carbonyl oxygen points towards the essential H176, which supports the role proposed for this residue along the two steps of the catalytic pathway. Arguments are provided that the structures reported here are representative of a productive enzyme/NAD/D-G3P complex in the ground state (Michaelis complex).
Introduction

Phosphorylating glyceraldehyde-3-phosphate dehydrogenases (GAPDH) are tetrameric enzymes that catalyse reversibly the oxidative phosphorylation of D-glyceraldehyde-3-phosphate (G3P) into 1,3 diphosphoglycerate (1,3 dPG) in the presence of cofactor NAD(P) via a two-step chemical mechanism. This mechanism has been well documented for the homotetrameric GAPDHs involved in glycolysis (1). First, the acylation step leads to formation of a thioacylenzyme intermediate and NADH. This includes 1) the binding of D-G3P to the binary complex GAPDH/NAD, 2) the formation of a covalent thiohemiacetal intermediate with D-G3P, and 3) the hydride transfer from the thiohemiacetal intermediate towards the C4 position of the nicotinamide ring of NAD. Second, the phosphorylation step consists of a nucleophilic attack of inorganic phosphate towards the thioacylenzyme intermediate that leads to formation of 1,3 dPG. This step, which is rate-limiting, includes the binding of inorganic phosphate to the enzyme intermediate possibly preceded by an isomerization step consisting of a replacement of NADH by NAD (2).

Two aminoacids are essential for the chemical mechanism, C149 and H176. The high catalytic efficiency of GAPDHs toward D-G3P implies prerequisites with respect to the chemical mechanism. First, C149 should be in a thiolate form within the ternary Michaelis complex to efficiently attack the C1 aldehydic group of G3P. This is ensured in part through an ion pair interaction with H176 which decreases C149 pKapp from 8.0 to 5.9 (3). Second, the hydride transfer should be efficient. This is assisted by H176 which plays a role as a base-catalyst. Moreover, the different intermediates and transition states, including the Michaelis complex GAPDH/NAD/D-G3P and the thioacylenzyme intermediate, should be stabilized. This is likely another role for H176 (Fig. 1).
The chemical nature of the substrates, G3P and inorganic phosphate, and the product, 1,3-dPG, implies the presence of two anion recognition sites in GAPDHs. These two anion binding sites have been tentatively identified within the active site from the location of two sulphate ions coming from the crystallisation medium (4). On the basis of model building of the lobster GAPDH thiohemiacetal intermediate, these two anion binding sites were postulated to correspond to those binding the C3-phosphate group of D-G3P (Ps site) and the inorganic phosphate ion (Pi site) (4). In all bacterial and eucaryotic GAPDHs, the Ps site is composed of the side chains of residues R231 and T179 and of the 2' hydroxyl group of the nicotinamide ribose when NAD is bound, and the Pi site includes the side chains of residues S148 and T208 and the main chain nitrogen of G209.

The Ps site has been found to occupy an identical location in all the crystal structures of eukaryotic and bacterial GAPDHs solved so far (4-14). But, the "true" location of the Pi site remains a matter of debate. Indeed, depending on the considered structure, the β strand-loop-α helix segment containing residues 206-210 (called loop 206-210) has been observed in two different conformations, giving rise to two alternative locations for the Pi site. The most common conformation is the one found in the holoenzyme from *B. stearothermophilus* (7) while a second conformation is observed for instance in the holostructure from *Leishmania mexicana* and generates a Pi site that is located closer to the catalytic C149 residue and 2.9 Å away from the former position (9).

The contribution of the Ps and Pi sites during the two steps of the catalytic mechanism remains also a matter of debate. Taking into account the revised orientation of the side chain of H176 in the refined holostructure of *B. stearothermophilus* GAPDH, Skarzynski et al. (7) noticed that it was no longer possible to build a reasonable model of the hemithioacetal with its C3-phosphate bound in the Ps site. They proposed that although the Pi site must be the location of the inorganic phosphate in the phosphorylation step, the C3-phosphate of the
substrate would bind first to the Pi site in the acylation step, and then flip from the Pi site to
the Ps site in the phosphorylation step. This hypothesis was supported by 1) the inspection of
the crystal structure of a ternary covalent complex GAPDH/NAD/glycidol-3-phosphate from
*B. stearothermophilus* which localizes the C$_3$-phosphate of the inhibitor within the Pi site (7)
and 2) also by kinetic analyses showing that substitution of R231 of the Ps site with glycine or
leucine does not significantly affect the first-order rate constants of the overall oxidoreduction
step (15) while mutations in the Pi site decrease this constant 4 to 7 fold (mutant T150A and
T208A, respectively. See (16))

The recent structure of a binary complex between *Escherichia coli* apoGAPDH and G3P, in
which the C$_3$-phosphate group of the substrate is bound in the so-called "new Pi site", also
supports this hypothesis (17). In the present study, the crystal structures of two mutant
GAPDHs from *B. stearothermophilus* in complex with the physiological substrate, D-G3P
have been determined at 2.0-2.2 Å resolution with the aim to evaluate the different hypotheses
on the contribution of both Ps and Pi sites along the catalytic pathway. For this purpose, the
active site C149 residue has been substituted by Ala and Ser. The C149A mutant GAPDH is
inactive while the C149S mutant GAPDH possesses a low residual activity (18). New

crystallisation conditions have been set up in the absence of any anions in order to avoid
competitive binding with the C$_3$-phosphate of D-G3P. The structures presented here constitute
the first example of ternary complexes mimicking the Michaelis complex wild-type
GAPDH/NAD/D-G3P. The results strongly suggest that the C$_3$-phosphate of D-G3P binds to
the Ps site at least in the Michaelis ternary complex.

**Experimental procedures**

*Site-directed mutagenesis, production and purification of mutant B. stearothermophilus GAPDHs*
Overexpression and purification of C149S mutant GAPDH were carried out by a similar procedure as described earlier (18). For C149A mutant GAPDH, overexpression was carried out in *E. coli* HB 101 strain (supE44, hsdS20 (r-, m-), recA13, ara-14, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1) transformed with a pBluescriptII-derivated plasmid containing the gapA gene of *B. stearothermophilus* under the control of the lac promoter. Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene). Purification of C149A mutant GAPDH was performed as described previously for other *B. stearothermophilus* mutant GAPDHs (3), except that a supplementary step was added in order to totally separate the *B. stearothermophilus* mutant GAPDH from the wild-type *E. coli* GAPDH produced by the HB101 cells. For this, enzyme solution was applied onto a Phenyl-Superose HR column (Amersham Pharmacia Biotech) equilibrated with Tris-HCl 50 mM, EDTA 2 mM, (NH₄)₂SO₄ 1.7 M, pH 8 buffer, followed by a linear gradient from 1.7 to 0.7 M of (NH₄)₂SO₄. Under these conditions, C149A mutant *B. stearothermophilus* GAPDH was eluted at 1070 mM of (NH₄)₂SO₄, and *E. coli* GAPDH at 850 mM.

Both mutant GAPDHs were isolated as apoform, as judged by the ratio of A₂₈₀/A₂₆₀ of 2. Purity of the enzymes was checked by SDS-PAGE and by mass spectrometry.

**Activity assay of GAPDH**

Initial rate measurements were carried out at 25°C on a Kontron Uvikon 933 spectrophotometer by following the absorbance of NADH at 340 nm. Thermostated sample holders using a circulating water bath for all the measurements maintained the temperature of the solutions at 25°C. The experimental conditions were 1 mM D-L G3P and 1 mM NAD in 40 mM triethanolamine, 2 mM EDTA, 50 mM K₂HPO₄, pH 8.9 buffer. Protein concentrations were calculated using a molar extinction coefficient at 280 nm of 1.17.10⁵ M⁻¹.cm⁻¹. Activities were expressed per monomer in s⁻¹.
Crystallisation and data collection

Crystals of the binary complexes I and II (C149A GAPDH/NAD and C149S GAPDH/NAD) were obtained at 293 K using the hanging-drop vapor diffusion technique (19). Crystals of the C149A mutant protein were grown from a solution composed of 8% (W/V) polyethylene glycol (PEG) 4K, 0.1M sodium acetate buffer pH 4.6, 2 mM EDTA, 2 mM DTT, 1.5 mM NAD while the C149S mutant protein crystallised in quite similar conditions but in Tris-HCl buffer pH 7.5 and with 10% (W/V) PEG 4K as precipitating agent.

The ternary complexes I and II (C149A GAPDH/NAD/D-G3P and C149S GAPDH/NAD/D-G3P) were obtained by soaking the crystals for 10 minutes in the crystallisation solution containing 1.9 mM DL-G3P. The crystals were then flash-frozen in liquid nitrogen by using 25 % (v/v) 2-methyl-2,4 pentadiol as a cryoprotectant.

The four data sets were collected at 100 K on an area detector (DIP2030) with a Phi goniometer using CuKα radiation from a rotating anode generator (Nonius B.V. FR591 Model). The data sets were processed and scaled with the HKL suite (20). The binary complex I crystallised in space group P3121 with one homotetramer per asymmetric unit. The crystal of the monoclinic binary complex II belongs to space group C2 and contains two independent dimers in the asymmetric unit (Fig. 2). The crystals of binary complex I and of ternary complex I diffracted to 2.2 Å resolution, while the crystals of binary complex II and of ternary complex II diffracted to 2.1 Å and 2.0 Å respectively. The statistics of the data sets are summarised in Table 1.

Phasing and Refinement

The structure of the binary complexes were solved by molecular replacement (21) using the wild-type holo structure (PDB code : 1gdl) as the starting model and data from the 10 to 5 Å
resolution range. Cycles of refinement (CNS, (22)) alternated with manual rebuilding (TURBO-FRODO, (23)) and inclusion of higher resolution data were carried out to improve the models.

Finally, the ordered water molecules were added. Each peak contoured at 3σ in the Fo-Fc maps was identified as a water molecule, provided that hydrogen bonds would be allowed between this site and the protein. The models were then adjusted and refined until a final convergence, using all the reflections (sigma cut-off = 0.0) with R and R-free values of 17.7 % and 21.0 % respectively for the C149A binary complex and 19.8% and 24.9% for the C149S binary complex (Table 1).

The ternary complexes were refined from the final models of the binary complexes using an identical procedure. The R and R-free values were 18.0 % and 21.4 % respectively for the C149A ternary complex, and 19.3 % and 23.5 % for the C149S ternary complex (Table 1).

Structure analyses and final structure parameters

The geometry of the models was checked with PROCHECK (24). Statistics concerning the geometry of the final models are given in Table 1. All non-glycine residues are located in favourable regions of the Ramachandran plot (25) except D186 and V237, which belong to external loops and whose side-chains are very well defined in the (2Fo-Fc) density maps. The location of these residues outside the allowed regions has already been noticed in the crystal structures of GAPDHs isolated from other sources (11). The coordinates and the structure factors of the binary and ternary complexes have been deposited to the protein data bank at Research Collaboratory for Structural Bioinformatics (PDB ID 1NPT, 1NQ5, 1NQA and 1NQO). Figures 2 to 5 were drawn with MOLMOL (26) or TURBO-FRODO (23).
Results

Biochemical properties of mutant GAPDHs

The *B. stearothermophilus* C149A mutant GAPDH was overproduced in *E. coli* cells, purified and separated from the *E. coli* GAPDH by taking advantage of its lower hydrophobicity. This separation was accompanied by a drastic diminution of the activity, from $5 \times 10^{-4}$ s$^{-1}$ to less than $1 \times 10^{-6}$ s$^{-1}$. This result clearly shows that the C149A mutant GAPDH displays no significant dehydrogenase activity in contrast to the *E. coli* C149A mutant GAPDH, which was shown to display a significant non-phosphorylating activity (27). This suggests subtle structural differences between the active sites of *E. coli* and *B. stearothermophilus* GAPDHs (see below). On the other hand, the C149S mutant GAPDH displayed, as already published (18), a low but significant phosphorylating dehydrogenase activity with a $k_{cat}$ of $8 \times 10^{-3}$ s$^{-1}$.

Overall structures

All GAPDH structures solved so far share a common conserved fold composed of a NAD(P) binding domain and of a catalytic domain. The enzyme is active as a homotetramer whose four subunits designated O, P, Q and R are related by three perpendicular 2-fold axes P, Q and R (for nomenclature, see (28)), the choice of the first monomer for denomination “O” being arbitrary. Since the crystals of the wild-type holoenzyme from *B. stearothermophilus* (7) had been obtained in the presence of ammonium sulphate that could have competed with the substrate for the binding to the anion recognition sites, new crystallisation conditions had to be set up for the present study. Two new crystal forms have been obtained using polyethylene glycol (PEG) 4K as the precipitant. The crystals of the binary complex C149A GAPDH-NAD (binary complex I) are trigonal and contain one typical homotetramer per asymmetric unit.
The asymmetric unit of the monoclinic crystals obtained from the binary complex C149S
GAPDH-NAD (binary complex II) is composed of two independent dimers (Fig. 2). Although
having a different orientation, these two dimers are equivalent and were thus called OQ and
O'Q'. Two biological tetramers (called A and B) were generated from these dimers OQ and
O'Q', by using the crystallographic 2-fold axis. In the tetramer A, the crystallographic axis is
along the 2-fold axis P, while in the tetramer B, it corresponds to the R axis. A striking feature
of this monoclinic form is the high compactness of the tetramers in the unit cell ($V_M : 2.19$
Å$^3$/Da) when compared to the trigonal form ($V_M : 2.92$ Å$^3$/Da).

No significant structural differences are observed between the four monomers within each
asymmetric unit. In the binary I and binary II complexes, the mean rms values obtained from
the different pairwised superimpositions of the C$_\alpha$ atoms are 0.26 Å and 0.30 Å, respectively.
For the ternary I and ternary II complexes, these values are 0.26 Å and 0.31 Å, respectively.
In the four monomers of the binary or ternary complexes, the NAD molecule is well defined
in the (2Fo - Fc) electron density maps (Fig. 3) and possesses average temperature factors
similar to those of the protein (Table 1).

Structure of the binary complexes: comparison with the wild-type holoenzyme

Since the structure of the wild-type holoenzyme revealed a quasi perfect 222 symmetry (7),
the least-square superimpositions involving the 334 C$_\alpha$ atoms of the subunit O of the wild-
type holoenzyme were realised with each subunit of the two binary complexes. The resulting
rms differences are less than 0.45 Å, showing that the overall structures of the binary I and
binary II complexes are very close to that of the wild-type holoenzyme. The largest
differences mainly concern either solvent exposed loops or regions involved in different
contacts with symmetry-related molecules. In addition, differences can be noted for the loop
206-210. This loop is located in the vicinity of the Pi site, which is "empty" in both binary I or
binary II complexes, while the side chain of T208 and the main chain nitrogen of G209 are
directly involved in the binding of a sulphate anion in the structure of the wild-type
holoenzyme (7). The displacement of residues 206-210 range from 0.2 to 1.1Å depending on
the subunit. Furthermore, the average temperature factors calculated for all the atoms of the
loop is 5 Å² higher than the average B factor calculated for all the atoms of the subunit. These
differences in B values are not observed in the wild-type holostructure, in which the presence
of the sulphate anion bound at the Pi site could stabilise the conformation of this loop.
Nevertheless, the amplitude of the displacement remains small and non significant when
compared with that observed for the *E. coli* GAPDH/G3P binary complex (17). In the present
work, the main conformation of the loop 206-210 is rather similar to that observed in the
structure of *B. stearothermophilus* wild-type GAPDH (7), as confirmed by omit maps (not
shown) calculated in this region. Therefore, the Pi site must still be structurally competent to
bind sulphate (or phosphate), even if the Pi site does not contain any sulphate anion in the
mutant structures, due to the crystallisation conditions used in this study.

In both binary complexes, a broad electron density peak, identified as a sulphate ion, is
observed within the Ps site. Since the crystallisation trials have been conducted in the absence
of sulphate, this anion arises most probably from the ammonium fractionation step of the
purification and has not been eliminated despite the extensive dialyses carried out prior to the
crystallisation experiments.

**Structures of the ternary complexes of mutant GAPDHs**

The comparisons of each ternary complex with its corresponding binary complex give rms
differences of less than 0.12Å showing that no significant conformational changes have been
induced upon G3P binding. The analysis of the maps (Fo-Fc) of the two ternary complexes clearly revealed a peak of electronic density corresponding to the substrate D-G3P (Fig. 4).

The substrate adopts the same conformation in both structures and shares almost the same interactions with the enzyme (Table 2). It is bound in a non covalent way, its C₃-phosphate being positioned within the Ps site (Fig. 5), while the Pi site is devoid of density. The oxygen atoms of C₃-phosphate group form hydrogen bonds with the conserved side chains of the residues R231 and T179 of the Ps site, and with the 2’ hydroxyl group of the ribose adjacent to the nicotinamide of NAD (Table 2, Fig. 5). In the holostructure of the wild-type enzyme (7), the non-conserved R195 residue of *B. stearothermophilus* GAPDH was also shown to interact with the sulphate anion at Ps site via a water molecule. This water molecule is present in the ternary complex I (wat 440, see Table 2) but is not found in the ternary complex II, although R195 occupies the same position than in the wild-type structure and in the ternary complex I. Nevertheless, in both ternary complexes, the direct interactions shared between the phosphate group of the substrate and the protein are exactly the same than those observed for the sulphate ion occupying the Ps site in the holostructure of the wild-type enzyme (7).

Whereas D,L-G3P was used for the soaking experiments, the C₂ carbon adopts an R configuration, which accounts for a selective binding of the D-G3P, as expected since D-G3P is a better substrate than L-G3P (29). The C₂ hydroxyl group of D-G3P points towards the NH main chain of residue 149, with a distance of 3.6 Å between G3P-O₂ and S (or A)149 NH (table2). The C₂ hydroxyl group also interacts with a water molecule (wat621), in the four monomers, which is stabilised through hydrogen bonding to the 2'O of the ribose adjacent to the nicotinamide ring (Fig. 5).

Although the equilibrium between the aldehyde and the gem-diol forms is strongly shifted in favour of the hydrated form in solution, analysis of the electron density allows us to conclude
unambiguously that the substrate is bound under its reactive form (free aldehyde) within the active site, even for the C149A mutant GAPDH. This result correlates with the lack of activity of the *B. stearothermophilus* C149A mutant GAPDH, but is nevertheless quite surprising because the C149A mutant GAPDH from *E. coli* was shown to use the hydrated form as a substrate (27). Therefore, one could have expected to observe the gem-diol form bound into the active site in the case of the ternary complex I even at the pH of 4.6 of the crystallisation medium. Some subtle differences in the active site between the *E. coli* and *B. stearothermophilus* C149A mutant GAPDHs probably account for this different behaviour, which remains however unexplained since the residues composing the active-site and their geometry are conserved in the two wild-type proteins (11). In the case of the ternary complex II of C149S mutant GAPDH, the lack of a covalent bond between D-G3P and S149 is consistent with the kinetic properties of the mutant protein. Indeed, the rate of the acylation step of which the nucleophilic attack of S149 was shown to be rate-limiting is 10^5-fold decreased compared to that of the k_{obs} of the wild type enzyme (18). In the crystallisation medium which contains PEG 4K, the acylation rate is probably even more decreased than in solution. This likely explains why under the experimental X-ray conditions which include a flash-freezing after 10 min of soaking, the non-covalent ternary complex remains chemically stable within the crystal.

**Discussion**

With the aim to understand better the catalytic mechanism of GAPDH and to evaluate the hypothetical models deduced from kinetic and structural studies, we solved the crystal structure of the *B. stearothermophilus* GAPDH in complex with its physiological substrate, D-G3P. For this purpose, the active site nucleophilic residue, C149, was substituted by alanine or serine. The mutant GAPDHs used here are either inactive (A149) or possess a low
residual activity (S149). New crystallisation conditions have been set up in the absence of any anions in order to avoid competitive binding with the C₃-phosphate group of the substrate. The ternary complex structures have been solved and constitute the first example of a ternary complex GAPDH/NAD/D-G3P. The substrate presents the same conformation in both structures. It is bound in a non-covalent way, under its aldehydic form, with its C₃-phosphate located in the Ps site. The binding of the C₃-phosphate group in the Ps site rather than in the Pi site is fully consistent with the observation that in the binary complexes, the Ps site was occupied with a sulphate ion coming from the purification step, whereas the Pi site was empty. This suggests a higher affinity of Ps site versus Pi site for phosphate and has been already observed in the structure of *B. stearothermophilus* wild-type enzyme since in this structure, the Ps site was fully occupied with sulphate coming from the crystallisation medium while the occupancy of the Pi site was only partial. This also supports the hypothesis of Chakrabarti (30) and Copley and Barton (31) that the presence of an arginine residue within an anion-binding site enhances its affinity for anions. In this regard, examination of the residues lining Ps and Pi sites shows that R231 is involved in the formation of the Ps site, while no arginine residue contributes directly to the Pi site. Electrostatic potential calculations, carried out on the monomer structure, confirmed that the local positive electrostatic potential is higher for Ps than for Pi sites (results not shown) and could explain the affinity differences between these two anion-recognition sites.

Whereas a racemic mixture of G3P was used for the soaking experiments, only the D-enantiomer is bound. The molecular factors explaining this stereoselectivity of GAPDHs have been the subject of literature controversies. Modelling the hemithioacetal intermediate within the active site of the lobster enzyme led Moras et al. (4) to propose that the preference for the D isomer could be explained by an interaction of C₂-hydroxyl group of the substrate with the
side chain of S148. This hypothesis had been then refuted by Skarzynski et al. (7) who
observed in the refined holostructure of \textit{B. stearothermophilus} enzyme the presence of a
hydrogen bond between the OH group of S148 and the main chain NH group of T151,
excluding thus the involvement of S148 in the stereoselectivity. More recently, Duée et al.
(11) modelled the hemithioacetal intermediate in the crystal structure of \textit{E. coli} GAPDH and
proposed that an interaction between the O$_2$ atom (Fig. 4) and the NH group of C149 would
explain this stereoselectivity. In our complexes, the side chain of S148 forms a hydrogen bond
with the amide group of T151, excluding any contribution of S148 to the stereoselectivity of
substrate. The distance between the C$_2$-hydroxyl group of D-G3P and the NH group of
residue 149 is consistent with the interpretation of Duée et al. (11). Furthermore, the
interaction between the C$_2$-OH and a water molecule, stabilised through hydrogen bonding to
the 2'O of the ribose adjacent to the nicotinamide ring (Fig. 5), might also contribute to the
stabilisation of the C$_2$-hydroxyl group.

In both ternary structures, the relative position of residue 149 with respect to the C$_1$ atom of
the substrate suggests that, assuming a similar positioning of C149, the nucleophilic attack of
C149 in the wild-type will occur on the Re face of the D-G3P. The carbonyl O$_1$ oxygen of D-
G3P is orientated for both mutant GAPDHs in such a way that it can accept an hydrogen bond
from the N$_{\varepsilon 2}$ atom of the catalytic H176 residue and also from the side-chain of S149 for the
ternary complex II (table 2). This is consistent with the role postulated for H176 in the
stabilisation of the substrate (3) (Fig. 1). Altogether, our results suggest that the structures of
the ternary complexes presented here are representative of the productive
enzyme/NAD/substrate complex in the ground state (Michaelis complex). Indeed, the use of a
C149S mutant GAPDH displaying a $K_M$ value for D-G3P similar to that of the wild-type and
the ideal orientation of the carbonyl O$_1$ oxygen with respect to the essential H176 residue let
us suppose that the substrate is bound in a catalytically competent manner, analogous to the one it adopts in the Michaelis complex in the case of the wild-type enzyme. These results, suggesting that the Ps site constitutes the binding site for the C3-phosphate group of the substrate, are however a bit surprising because former structural studies carried out with the aim to obtain a structure that mimics the hemithioacetal intermediate pointed to the involvement of the Pi site. Indeed, the structure of *B. stearothermophilus* holoenzyme in complex with a substrate analogue, glycidol-3-phosphate (7), as well as the structure of *E. coli* apoGADH in complex with G3P (17), both revealed that the C3-phosphate group was located in the Pi site. From this point of view, the structures of the ternary complexes presented here are in apparent contradiction with the structural studies and with the biochemical results mentioned in the introduction. Several reasons - which are not mutually exclusive- can be put forward to explain this apparent discrepancy.

i) First of all, the structure of the *E. coli* enzyme in complex with G3P was carried out on the apoform (without NAD). It is however well established that NAD contributes to the formation of the Ps site (7), and therefore, the lower affinity of a non-functional Ps site might explain why the C3-phosphate was found in the Pi site in this structure. Furthermore, for this latter structure, Yun et al. (17) noticed that the C1-hydroxyl group of G3P and the nicotinamide base would be close to each other, and proposed that the positive charge of the nicotinamidium contributes to the stabilisation of the tetrahedral transition intermediate. It was also observed that H176 was involved in the binding of the phosphate group of G3P, and thus would be unable to play a role in stabilising the tetrahedral intermediate and as base catalyst for facilitating hydride transfer, which does not correlate with the conclusions drawn from former site-directed mutagenesis experiments (3).

ii) Second, when compared now to the *B. stearothermophilus* GADPH/NAD/glycidol-3-phosphate structure, the use of the true substrate of the enzyme in our ternary complexes...
shows that an H-bond is formed between the C_1 carbonyl and H176-N_{ε2} that could explain the
different positioning of glycidol-3-phosphate. Indeed, the lack of oxygen atom at the C_1
position in the latter molecule prevents formation of such an H-bond.

iii) A third difference concerns the absence of a covalent link between the C_1 carbonyl
atom and the catalytic residue C149 in our complex when compared to the two structures
mentioned above.

In previous kinetic studies (15,16), changing Ps-site residues was shown to strongly decrease
the k_{cat} while that of Pi-site residues led to moderate k_{cat} effects. In all mutant GAPDHs, the
limiting step was shown to take place after the hydride transfer and the K_M values of G3P
were not drastically modified. The fact that changing Pi-site residues decreased the first-order
overall oxidoreduction rate while changing Ps-site residues did not, was interpreted as an
evidence in favor of an initial binding of the C_3-phosphate at the Pi site. Although it seems
probable from the current structures that the Ps site in the wild-type enzyme constitutes the
binding site for the C_3-phosphate group of the substrate in the non-covalent complex
GAPDH-NAD-G3P, our results do not rule out the possibility that the formation of the
covalent bond between C149 and G3P promotes the repositioning of the C_3-phosphate in the
Pi site before the hydride transfer. Modelling the hemithioacetal intermediate in the B.
stearothermophilus wild-type enzyme (not shown) with the C_3-phosphate group positioned
either in the Ps or in the Pi site leads to models equivalent to those already reported by Duée
et al. (11) or Kim et al. (9). It is furthermore obvious from these modelling experiments that
changing the position of the C_3-phosphate group from Ps site to Pi site requires only a rotation
around the C_1-C_2 bond. This rotation can be achieved without significant changes in the
position of the C_1 atom, and whatever the location of the C_3-phosphate group either in Ps or in
Pi site, leads to a complex in which the relative position of H176 with respect to the C_1-
hydroxyl group is consistent with the role of base-catalyst proposed for H176. Therefore, it is
not possible to use these models to discriminate between the two alternative positioning for
the C3-phosphate group at the hemithioacetal intermediate level, except that binding in the Ps
site seems to be more favourable from an energetic point of view, as already discussed by
Kim et al. (9).

To conclude, the structures of the ternary complexes presented here are probably
representative of that of the wild-type enzyme/NAD/D-G3P ternary complex in the ground
state. But it remains to know whether the substrate will keep the same position, in particular
with regard to its C3-phosphate group, when the covalent bond within the ternary complex is
formed between C149 and D-G3P. The fact that the thioacylenzyme intermediate can
accumulate in the absence of phosphate could allow the determination of its crystal structure
and thus the localisation of the C3-phosphate within one of the different covalent complexes
formed along the catalytic mechanism.

Acknowledgments

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Ministère de la Recherche et de l’Enseignement Supérieur and local funds of the Région
Lorraine. We are very grateful to Dr S. Azza for the production and purification of enzymes
and to Drs A. Van Dorsselaer and S. Sanglier-Cianferani for the mass spectrometry analyses. We
would like to thank the Service Commun de Diffraction X sur Monocristaux (Université
Henri Poincaré-Nancy 1) for providing access to crystallographic experimental facilities.
References


**Fig. 1. Schematic representation of the postulated catalytic mechanism of phosphorylating GAPDHs.** The catalytic mechanism can be divided into two steps. In the acylation step, C149 and H176 form an ion pair in holo-GAPDH (a). This decreases the pK<sub>app</sub> of C149 thus facilitating the thiolate attack toward the C<sub>1</sub> of D-G3P. The role of H176 is also to stabilize the binding of 1) the substrate in the Michaelis complex GAPDH/NAD/D-G3P (b), 2) the thiohemiacetal intermediate (c) and 3) the thioacylenzyme intermediate (d). H176 also plays a role as a base catalyst facilitating the hydride transfer from the thiohemiacetal toward the nicotinamidium of NAD (c). In the phosphorylating step, binding of inorganic phosphate to the thioacyl enzyme is followed by its nucleophilic attack toward the thioacyl intermediate (d) which leads, via a sp<sup>3</sup> phosphorylated intermediate (e), to formation and release of 1,3-dPG (f). H176 is postulated to stabilize the tetrahedral intermediate (e) and to facilitate as acid (d) and base (e) catalysts the 1,3-dPG formation. The exchange cofactor step which consists of NADH release prior to NAD and inorganic phosphate binding remains controversial (32-40). R' represents the adenine-ribose-phosphate-phosphate-ribose part of the cofactor NAD. R represents the CH(OH)COPO<sub>3</sub> part of the substrate D-G3P.

**Fig. 2. Crystal packing of the monoclinic form.** The two independent dimers of the asymmetric unit are represented in grey. Their symmetry-related dimers are shown in black. The symmetry operators (-x, y, 1-z) and (2-x, y, 1-z) were used to generate tetramer A, and tetramer B respectively. In this stereoview, the 2-fold crystallographic axis is vertical and corresponds to the P axis for the tetramer A and to the R axis for the tetramer B.

**Fig. 3. Stereoview of the final (2fo-fc) electron density map of the cofactor NAD<sup>+</sup>.** This view corresponds to monomer O of the ternary complex I (contour level: 1.2 σ).
**Fig. 4. Views of the D-G3P molecule** A) View of the D-G3P molecule with its atom numbering and B) Stereoview of the D-G3P molecule in the monomer O of the ternary complex I shown in the (Fo-Fc) electron density calculated from the refined structure before the introduction of the substrate (contour level : $3 \sigma$).

**Fig. 5. Stereoview of the active site in the monomer O of the ternary complexes** A) ternary complex I (C149A mutant GAPDH) and B) ternary complex II (C149A mutant GAPDH). The residues that interact with the G3P molecule are labelled.
Table 1: Data collection and refinement statistics

<table>
<thead>
<tr>
<th>Crystal</th>
<th>Binary complex I</th>
<th>Ternary complex I</th>
<th>Binary complex II</th>
<th>Ternary complex II</th>
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<tbody>
<tr>
<td><strong>A. Data Collection</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
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<td>Space group</td>
<td>P3_{1}21</td>
<td>P3_{1}21</td>
<td>C2</td>
<td>C2</td>
</tr>
<tr>
<td>a (Å)</td>
<td>115.7</td>
<td>115.6</td>
<td>140.48</td>
<td>140.31</td>
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<tr>
<td>b (Å)</td>
<td>115.7</td>
<td>115.6</td>
<td>87.91</td>
<td>87.84</td>
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<tr>
<td>c (Å)</td>
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<td>223.9</td>
<td>119.92</td>
<td>119.41</td>
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<tr>
<td>β°</td>
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<td></td>
<td>119.0</td>
<td>119.1</td>
</tr>
<tr>
<td>Z</td>
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<td>6</td>
<td>4</td>
<td>4</td>
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<td>Nominal resolution (Å)</td>
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<td>Outermost resolution shell (Å)</td>
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<td>2.28-2.2</td>
<td>2.18-2.11</td>
<td>2.07-2.01</td>
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<td>110</td>
<td>110</td>
<td>110</td>
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<td>Unique reflections</td>
<td>85949</td>
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<td>77562</td>
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<tr>
<td>Completeness (%)</td>
<td>96.1 (91.)</td>
<td>96.3 (98.9)</td>
<td>99.2 (75.6)</td>
<td>92.8 (74.8)</td>
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<tr>
<td>R-merge¹</td>
<td>0.061 (0.16)</td>
<td>0.065 (0.29)</td>
<td>0.085 (0.28)</td>
<td>0.105 (0.32)</td>
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<tr>
<td>Mean I/s(I)</td>
<td>16.1 (5.2)</td>
<td>13.3 (3.7)</td>
<td>14.0 (3.4)</td>
<td>12.1 (3.2)</td>
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<td><strong>B. Refinement</strong></td>
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<tr>
<td>R-factor² (%)</td>
<td>17.7(20.8)</td>
<td>18.0(20.4)</td>
<td>19.8(25.4)</td>
<td>19.3(23.8)</td>
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<tr>
<td>R-free³ (%)</td>
<td>21.0(24.1)</td>
<td>21.4(25.1)</td>
<td>24.9(31.5)</td>
<td>23.5(31.5)</td>
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<tr>
<td>r.m.s.d. from ideal geometry:</td>
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<td>Bond lengths (Å)</td>
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<td>0.005</td>
<td>0.005</td>
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<td>1.4</td>
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<td>Average B factor (Å²)</td>
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<td>17.4</td>
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<td>NAD</td>
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<td>16.7</td>
<td>19.4</td>
<td>11.7</td>
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<td>Sulphate</td>
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<tr>
<td>G3P</td>
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<tr>
<td>Water molecules</td>
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<td>29.31</td>
<td>28.3</td>
<td>22.6</td>
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¹R-merge: R-factor for symmetry related intensities. ²R-factor: crystallographic R-factor. R-
free³: R-factor for a randomly selected 10% of reflections not included in refinement. Values
between brackets refer to the outermost resolution shell.
Table 2: Interactions between the substrate, D-G3P, and the active site residues.

<table>
<thead>
<tr>
<th>Ternary complex I (C149A mutant GAPDH)</th>
<th>O</th>
<th>P</th>
<th>Q</th>
<th>R</th>
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<tr>
<td>G3P-O4P --- wat 353</td>
<td>2.54</td>
<td>2.61</td>
<td>2.73</td>
<td>2.68</td>
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<tr>
<td>G3P-O4P --- O2'N-NAD</td>
<td>3.12</td>
<td>3.14</td>
<td>3.19</td>
<td>3.19</td>
</tr>
<tr>
<td>G3P-O2P --- T179-OG1</td>
<td>2.50</td>
<td>2.57</td>
<td>2.51</td>
<td>2.50</td>
</tr>
<tr>
<td>G3P-O2P --- R231-NH2</td>
<td>2.76</td>
<td>2.89</td>
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<tr>
<td>G3P-O3P --- wat 440</td>
<td>2.92</td>
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<td>2.99</td>
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<td>G3P-O2 --- wat 621</td>
<td>2.52</td>
<td>2.75</td>
<td>2.61</td>
<td>2.50</td>
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<tr>
<td>G3P-O2 --- A149N</td>
<td>3.58</td>
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<td>3.70</td>
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<tr>
<td>G3P-O1 --- H176NE2</td>
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<td>2.69</td>
<td>2.74</td>
<td>2.79</td>
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<table>
<thead>
<tr>
<th>Ternary complex II (C149S mutant GAPDH)</th>
<th>O</th>
<th>Q</th>
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<th>Q'</th>
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<td>G3P-O4P --- wat 353</td>
<td>2.60</td>
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<td>2.56</td>
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<td>G3P-O2P --- T179-OG1</td>
<td>2.44</td>
<td>2.53</td>
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<td>2.74</td>
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<tr>
<td>G3P-O2P --- R231-NH2</td>
<td>2.64</td>
<td>2.74</td>
<td>2.66</td>
<td>2.64</td>
</tr>
<tr>
<td>G3P-O2 --- wat 621</td>
<td>2.55</td>
<td>2.75</td>
<td>2.93</td>
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<tr>
<td>G3P-O2 --- S149N</td>
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<td>3.66</td>
<td>3.60</td>
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<tr>
<td>G3P-O1 --- S149-OG</td>
<td>2.37</td>
<td>2.43</td>
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<td>2.39</td>
</tr>
<tr>
<td>G3P-O1 --- H176NE2</td>
<td>2.60</td>
<td>2.51</td>
<td>2.48</td>
<td>2.65</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2
Figure 3
Figure 5
Crystal structure of two ternary complexes of phosphorylating glyceraldehyde-3-phosphate dehydrogenase from Bacillus stearothermophilus with NAD and D-glyceraldehyde-3-phosphate

Claude Didierjean, Catherine Corbier, Mustapha Fatih, Frédérique Favier, Sandrine Boschi-Muller, Guy Branlant and André Aubry

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