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To cite this version:

Sébastien Moniot, Stefano Bruno, Clemens Vonrhein, Claude Didierjean, Sandrine Boschi-Muller, et al.. Trapping of the Thioacylglyceraldehyde-3-phosphate Dehydrogenase Intermediate from Bacillus stearothermophilus. Journal of Biological Chemistry, American Society for Biochemistry and Molecular Biology, 2008, 283 (31), pp.21693-21702. 10.1074/jbc.M802286200. hal-01690593

HAL Id: hal-01690593
https://hal.univ-lorraine.fr/hal-01690593
Submitted on 23 Jan 2018

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Trapping of the Thioacylglyceraldehyde-3-phosphate Dehydrogenase Intermediate from Bacillus stearothermophilus

DIRECT EVIDENCE FOR A FLIP-FLOP MECHANISM

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The crystal structure of the thioacyl enzyme intermediate of the phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Bacillus stearothermophilus has been solved at 1.8 Å resolution. Formation of the intermediate was obtained by diffusion of the natural substrate within the crystal of the holoenzyme in the absence of inorganic phosphate. To define the soaking conditions suitable for the isolation and accumulation of the intermediate, a microspectrophotometric characterization of the reaction of GAPDH in single crystals was carried out, following NADH formation at 340 nm. When compared with the structure of the Michaelis complex (Didierjean, C., Corbier, C., Fatih, M., Favier, F., Boschi-Muller, S., Branlant, G., and Aubry, A. (2003) J. Biol. Chem. 278, 12968–12976) the 206–210 loop is shifted and now forms part of the so-called “new Pi” site. The locations of both the O1 atom and the C3-phosphate group of the substrate are also changed. Altogether, the results provide evidence for the flipping of the C3-phosphate group occurring concomitantly or after the redox step.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a homotetrameric enzyme catalyzing the oxidative phosphorylation of d-glyceraldehyde 3-phosphate (G3P) into 1,3-bisphosphoglycerate (1,3-DPG), in the presence of inorganic phosphate (Pi) and nicotinamide adenine dinucleotide (NAD+).

The reaction mechanism has been intensively investigated in particular for bacterial and eukaryotic GAPDHs (1–9) and consists of two steps as follows: (i) an oxidoreduction reaction, corresponding to the nucleophilic attack of the catalytic cysteine (Cys-149) on the aldehydic group of G3P, followed by a hydride transfer assisted by His-176 (base catalyst) from the generated thiohemiacetal to the C4 of the nicotinamide of NAD+ that leads to the formation of a thioacyl enzyme (7), and (ii) a phosphorylation of the resulting thioester through the nucleophilic attack of inorganic phosphate on the carboxyl group of the thioacyl enzyme. The second step is preceded by the exchange of NADH with NAD+, with the latter favoring the phosphorolysis step.

Two anion recognition sites accommodate the inorganic phosphate ion and the phosphate groups of G3P and 1,3-DPG. Their positions within the active site have been deduced from the location of two sulfate ions derived from the ammonium sulfate crystallization medium (9). On the basis of a model of the thiohemiacetal intermediate in the Homarus americanus GAPDH structure, the anion binding sites were initially attributed to the specific binding of the C3-phosphate (C3P) group of d-G3P (Psite) and of the inorganic phosphate ion (Pj) site. The location of the Pj site in the three-dimensional structures of eukaryotic and bacterial GAPDHs is conserved and independent of the enzyme state, apo-, or holo-form, and of the presence of ligands such as sulfate ions, phosphate ions, substrate, or substrate analogs. This Ps site is composed of the side chains of residues Arg-231 and Thr-179 and the 2′-hydroxyl group of the nicotinamide ribose of NAD+. On the contrary, the location of the Pj site appears to vary depending on the presence and nature of the bound ligands or source organism. This location is related to the conformation adopted by the segment composed of residues 206–212. Although the most common conformation is that originally found in the holoenzyme...
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from H. americanus and Bacillus stearothermophilus (9–10), an alternative conformation was first observed in the holoenzyme from Thermotoga maritima and Leishmania mexicana (11–12) that generates a P_i site located closer to the catalytic Cys-149 residue and 3 Å away from the former position, which has been called new P_i site. Whatever the considered position, the P_i site includes the side chains of residues Ser-148 and Thr-208 and the main-chain nitrogen of Gly-209. In addition, the new P_i site also involves residue Thr-150.

Despite numerous functional (6, 7, 13) and structural studies (14–16), the individual role of P_i and P_s sites in the catalytic events remains unclear. Although it is generally assumed from geometrical considerations that inorganic phosphate must attack the intermediate from the P_i site, a possible flipping of the C3P of G3P between the two sites during catalysis was suggested by Skarzynski et al. (10).

Recently, using mutants of the active site cysteine, Didierjean et al. (16) obtained the structures of ternary complexes from B. stearothermophilus GADHP with NAD$^+$ and the physiological substrate D-G3P, the phosphate group of which was found to be bound at the P_s site. This result strongly suggests that the P_s site constitutes the binding site for the C3P group of the substrate but does not, however, exclude the possibility that the substrate could display different interactions especially with regard to its C3P group, once the covalent bond is formed between Cys-149 and D-G3P. Indeed this structure is representative of a Michaelis, non-covalent complex and does not allow to prefigure the location of the C3P group in the intermediate stages of the reaction.

Much of the uncertainty regarding the individual contribution of the two anion binding sites during the catalytic events derives from the absence of the structure of the physiological thioacyl enzyme intermediate. To obtain this structure, holo-GAPDH crystals from B. stearothermophilus were soaked with the substrate G3P in the absence of inorganic phosphate. Formation of NADH in the crystal was monitored by single crystal absorption microspectrophotometry (17), a method pioneered by Rossi and Bernhard (18), for the determination of protein function in the crystalline state (19) and for the design of kinetic crystallography experiments (20). A series of microspectrophotometric studies on GAPDH crystals were previously carried out using a chromophoric substrate analog of 1,3-DPG (21–23). The experimental conditions suitable for the accumulation of the catalytically-relevant thioacylenzyme intermediate in the crystals of B. stearothermophilus GAPDH were thus designed and allowed us to solve its structure. This constitutes the first structure of a reaction intermediate of GAPDH in the presence of its cofactor NAD$^+$. Analysis of the acylated structure reveals two major outcomes when compared with that of the non-covalent complex: a shift of the 206–210 loop, which delineates a new position for the P_i site comparable to that originally described for the holo-structure of Thermotoga maritima, and the location of the C3P group of the thioacyl intermediate, which is bound in this new P_i site.

EXPERIMENTAL PROCEDURES

Chemicals—DL-Glyceraldehyde-3-phosphate (DL-G3P, Sigma) was prepared from the DL-G3P diethyacetal according to the manufacturer, and its concentration was assessed using GAPDH. Iodoacetic acid, ammonium sulfate, EDTA, NAD$, Tris, and dithiothreitol were from Sigma and were of the best available quality and used without further purification.

Production, Purification, and Crystallization of Wild-type B. stearothermophilus GAPDH—The Escherichia coli strain used for wild-type GAPDH production was HB101 transformed with a pBluescript II SK containing the gap gene under the lac promoter. Purification was performed as previously described by Talfournier et al. (7). Purity of the enzyme was checked by electrophoresis on a 10% SDS-PAGE and by mass spectrometry.

Prior to crystallization, the enzyme was dialyzed overnight against 100 mM Tris buffer, pH 8.0, 2 mM EDTA, and 2 mM dithiothreitol, and concentrated using a 30-kDa molecular mass cut-off centrifugation system (Centricon). Protein concentration was determined spectrophotometrically at 280 nm using a molecular mass of 143,776 Da and an extinction coefficient of 1.31 $\times$ 10$^5$ M$^{-1}$ cm$^{-1}$.

Crystals of the holo-form of wild-type B. stearothermophilus GAPDH were obtained at 293 K using the hanging-drop, vapor diffusion method in the presence of ammonium sulfate as precipitating agent. The reservoir solution contained 2.7 M ammonium sulfate, 100 mM Tris-HCl buffer, pH 6.9. Drops were prepared by mixing in different ratios a solution containing 15 mg ml$^{-1}$ enzyme and 2 mM NAD$^+$ and the reservoir solution. Crystals appear within 1 week from drops with mixed volumes of protein and reservoir solutions in ratios of 1/2, 1/1, or 2/1.

Single Crystal-polarized Absorption Microspectrophotometric Measurements—Single crystals of GAPDH were resuspended at least six times in a stabilizing solution containing 2.7 M ammonium sulfate, 0.1 M Tris-HCl buffered at pH 8.2. Crystals were loaded in a quartz flow cell mounted on the stage of a Zeiss MPM03 microspectrophotometer, equipped with a 10X ultrafluar objective and a thermostatic apparatus (24). Polarized absorption spectra were recorded between 300 and 500 nm, with the electric vector of the linearly polarized light parallel to crystal edges.

To follow the reaction of GAPDH crystals with D-G3P, crystals were suspended in the stabilizing solution containing DL-G3P at concentrations up to 60 mM. Polarized absorption spectra were recorded as a function of time to monitor reaction equilibration. To obtain the reference spectrum for the fully reduced NADH-anion irreversibly reacts with the thioacylenzyme, shifting the equilibrium toward the full reduction of NAD$. At each G3P concentration, the fraction of reduced NADH was calculated by fitting the corresponding absorption spectrum to a linear combination of the spectra of the native form and the arsenate-treated form. As a control experiment, enzyme crystals were suspended in a solution containing 10 mM iodoacetic acid, 2.7 M ammonium sulfate, 0.1 M Tris-HCl buffer, pH 8.2. This experiment was aimed to demonstrate the disappearance of the Racker band, diagnostic of an active enzyme, due to the
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alkylation of the reactive Cys-149. All experiments were carried out at 15 °C.

**X-ray Diffraction Experiments**—Crystals of the holoenzyme were soaked for 1.5–10 min in a crystallization medium containing 60 mM G3P. Reaction was stopped by flash freezing crystals after a quick soak in a cryoprotective solution containing 20% (v/v) glycerol in addition to the crystallization medium. Several datasets were collected at 100 K using a synchrotron beam either at European Synchrotron Radiation Facility (Grenoble, France) or Deutsches Elektronen-Synchrotron (Hamburg, Germany). 360 frames each of 0.5° oscillation steps were systematically collected to assure collection of complete and redundant datasets, which were processed using the XDS package (25) and scaled using SCALA (26). The structure reported here (see below) derived from a crystal, soaked for 3 min, which diffraction data were collected at the X13 beamline (Deutsches Elektronen-Synchrotron) using a MARCCD detector (165 mm) and a wavelength of 0.806 Å. Statistics of the corresponding 1.77-Å resolution dataset are summarized in Table 1. Holo-crystals belong to monoclinic space group P2₁ with typical unit cell dimensions of a = 82 Å, b = 123 Å, c = 82 Å, and β = 111°, containing one physiological homotetramer per asymmetric unit (as previously described by Skarzynski et al. (10)).

**Phasing and Refinement**—The structure was solved by molecular replacement using the MOLREP program (27) of the CCP4 suite (28) with the wild-type holo-structure (Protein Data Bank code 1gd1 (10)) as a starting model and data up to 3.6-Å resolution. Cycles of refinement (Refmac5) alternated with manual rebuilding (Coot) were carried out to improve the model (29, 30). Water molecules were added using the built-in find-water function of Coot and individually checked for significant signal and consistent contact with H-bond donor/acceptor. The thioacylenzyme model obtained from soaked ammonium sulfate-grown crystal was refined up to final convergence, with all the reflections (sigma cut-off = 0) with R and Rfree of 18.2% and 23.9%, respectively. The final cycles of refinement were performed using the BUSTER-TNT program (31), which allowed refinement of occupancy factors of the catalytic cysteine, reaction intermediate, 206–212 loop, and sulfate anions within the four monomers. This gave a final R and Rfree of 16.1% and 19.8%, respectively.

**Structure Analysis and Final Structure Parameters**—The geometry of the model was checked with PROCHECK (32). Statistics concerning the geometry of the final model are given in Table 1. All non-glycine residues are located in favorable regions of the Ramachandran plot (33) except Asp-186 and Val-237, two residues already reported to be outside the allowed regions of the Ramachandran plot in the crystal structures of GAPDH isolated from *B. stearothermophilus* and other sources (34). The coordinates and structure factors of the thioacylenzyme structure have been deposited to the Protein Data Bank at Research Collaboratory for Structural Bioinformatics (ID code 3cmc). Figs. 2–4 were drawn with PyMOL.4

4 W. L. DeLano (2002) PyMOL, DeLano Scientific, San Carlos, CA.

**FIGURE 1.** Polarized absorption spectra of single crystals of *B. stearothermophilus* holo-GAPDH in the absence and presence of G3P and iodoacetic acid. GAPDH crystals, grown in ammonium sulfate, were soaked in a stabilizing solution containing 2.7 mM ammonium sulfate, 0.1 M Tris-HCl, buffered at pH 8.2 (solid line). The spectra were recorded with the light polarized perpendicular to the b axis of the crystal. The crystal was then resuspended three times with the same solution containing 10 mM iodoacetic acid (dashed line). Within the time required for the washing steps, a complete disappearance of the Racker band was observed. A different crystal was soaked with the stabilizing solution containing increasing concentrations of G3P (10, 15, 30, and 60 mM). The absorption spectrum obtained at 60 mM G3P is reported (dash-dot line). Further reduction of NAD⁺ was obtained by soaking enzyme crystals with the stabilizing solution containing 60 mM G3P and 1 mM sodium arsenate (dotted line). The fractional reduction of NAD⁺ by G3P was calculated at different G3P concentrations by fitting the observed spectra to a linear combination of the spectrum of the native form and the arsenate-treated form. Inset: dependence of the fractional reduction of NAD⁺ as a function of G3P concentration (10, 15, 30, and 60 mM). Spectra recorded on different crystals were normalized using the spectrum of the holoenzyme as reference.

**RESULTS**

Polarized Absorption Spectra of *B. stearothermophilus* Holo-GAPDH Crystals in the Absence and Presence of G3P—Polarized absorption spectra of holo-GAPDH crystals from *B. stearothermophilus* grown in ammonium sulfate exhibited a broad band centered at around 360 nm (Fig. 1). This spectral feature, known as the Racker band, is associated with a charge transfer between the catalytic cysteine residue and the NAD⁺ nicotinamide ring (35). Carboxymethylation of GAPDH crystals at the Sγ of Cys-149 with iodoacetic acid resulted in the disappearance of the band (Fig. 1), as in solution, indicating that GAPDH in the crystal is in a catalytically active conformation. A similar observation was obtained on GAPDH crystals from *Palinus vulgaris* (21).

Upon addition of the physiological substrate G3P to holoenzyme-NAD⁺ crystals, an intense band centered at around 340 nm, associated with the formation of NADH, appeared with the concomitant disappearance of the Racker band (Fig. 1). This result indicates that the redox reaction has occurred in the crystalline state, with the hydride transfer from C1 of G3P to C4 of nicotinamide ring and concom-
Table 1: Data collection and refinement statistics

| Data collection | Values
|-----------------|--------------------------------------------------|
| **Thioacyl enzyme structure** | (soaked ammonium sulfate crystal) |
| **Space group** | P2₁ |
| **Unit cell (Å)** | a = 81.7; b = 122.6; c = 81.8; β = 111.1 |
| **Z** | 2 |
| **Nominal resolution (Å)** | 1.77 |
| **Outermost resolution shell (Å)** | 1.88-1.77 |
| **Temperature (K)** | 100 |
| **Unique reflections** | 142,733 (20,259) |
| **Completeness (%)** | 97.7 (92.9) |
| **Redundancy** | 3.7 (3.2) |
| **Rmerge (%)** | 2.8 (18.9) |
| **Mean 1/σ(I)** | 32.1 (6.5) |

**Refinement**

| **R-factor (%)** | 16.1 |
| **Rmerge (%)** | 19.8 |
| **r.m.s.d. from ideal geometry** | 0.011 |
| **Bond angles (°)** | 1.27 |
| **Dihedral angles (°)** | 17.0 |
| **Improper angles (°)** | 26.0 |
| **Mean B-factor (Å²)** | 25.7 |
| **Overall model** | 23.6 |
| **NAD** | 17.1 |
| **Reaction intermediate** | 27.4 |
| **Sulfate atoms** | 40.4 (45.8-34.7) |
| **Water molecules** | 37.7 |

* R-factor for symmetry-related intensities.
* Crystallographic R-factor.
* Crystallographic R-factor for a randomly selected 5% of reflections not included in refinement.

Table 2: Occupancy factor refinement and thermal agitation values

| Subunit | O | P | Q | R |
|---------|---|---|---|---|
| Thioacyl intermediate | | | | |
| Refined occupancy | 0.64 | 0.59 | 0.62 | 0.67 |
| Mean B value (Å²) | 27.5 | 27.9 | 26.8 | 27.6 |
| Real space correlation coefficient* | 0.95 | 0.91 | 0.94 | 0.94 |
| Sulfate (P, site) | | | | |
| Occupancy | 0.33 | 0.47 | 0.41 | 0.27 |
| B value (Å²) | 46.1 | 45.6 | 45.1 | 46.3 |
| 206–212 loop (confA/confB) | | | | |
| Refined occupancy | 0.47/0.53 | 0.66/0.34 | 0.65/0.34 | 0.44/0.65 |
| Mean B value (Å²) | 24.8/25.6 | 24.5/25.2 | 23.8/24.5 | 24.9/25.5 |
| Real space correlation coefficient* | 0.93/0.87 | 0.95/0.90 | 0.95/0.89 | 0.94/0.88 |

* Correlation existing between model density and 2mFo − DFo density.

**Thioacyl enzyme intermediate from B. stearothermophilus GADPH**

Crystals of GADPH, grown from polyethylene glycol, and 1 molecule of ethylene glycol derived from the cryoprotective treatment (see “Experimental Procedures”) and 1743 water molecules. Except for some solvent-exposed side chains, residues of the model are well defined in the electron density maps. Only a few residual peaks, mostly in the solvent-exposed region, were not interpreted in the difference maps. The model has tight stereochemical restraints with r.m.s.d. on bond lengths and on bond angles of 0.01 Å and 1.27°, respectively (Table 1).

The four monomers are named O, P, Q and R, following the nomenclature initially described for H. americanus GADPH (37). Using the O subunit as a reference, the root-mean-square deviations (r.m.s.d.) of backbone atoms after superimposition of the P, Q, and R subunits are 0.15, 0.14, and 0.12 Å, respectively, showing that the conformations of all four subunits in the final refined tetramer are very similar. Superimposition of the backbone atoms of our model onto the holo-structure described by Skarzynski et al. (10) (same space group and unit cell dimensions; pdb code 1gd1) gives an r.m.s.d. of 0.45 Å for the whole tetramer and a mean value of 0.27 Å for the different pairwise superimpositions of the monomers. No major difference was observed between the two structures except locally for the 206–212 loop, which exhibits deviations three times higher than the r.m.s.d. This conformational difference will be further described below.

As expected from the stoichiometry of the reaction of GADPH crystals with 60 mM DL-G3P (fraction of NADH per monomer of ~0.70), determined by polarized absorption microspectrophotometry (Fig. 1, inset), the active site of each subunit was found to be present in two distinct, differently populated chemical states, which have been assumed to correspond to the native holo-structure with a free cysteine and a sulfate anion bound to the P₁ site (mean refined fractional occupancy of ~0.4, conformation A) and to the thioacylenzyme-NADH intermediate (mean refined fractional occupancy of ~0.6, conformation B) (Table 2). The refined occupancies and B values of the ligands are similar in the four monomers and support an equivalent distribution of the thioacyl enzyme intermediate in all subunits. The differences between the two states are also

**Overall Structure and Model Quality**—The final model is composed of one tetramer in the asymmetric unit, each monomer containing residues 0–333 and one molecule of cofactor. The asymmetric unit also contains 4 bound G3P molecules, 18 sulfate ions (including two in each active site), 8 molecules of glycerol, and 1 molecule of ethylene glycol.
reflected in the 206–212 loop that was built in the electron density maps in two alternate conformations, A and B, each associated with one of the two states presented above. Refinement of the occupancies of both 206–212 loop alternate conformations shows the existence of two subunit types. O and R subunits exhibit almost equal occupancies for the two conformations, whereas the native conformation is prevailing in P and Q (Table 2). Although this loop is not directly involved in crystalline packing, such difference could arise from the distinct crystalline environment of each subunit extensively described by Skarzynski et al. (10). Despite these slight occupancy variations, conformations of the 206–212 loop and of bound ligands are similar in the four monomers. Therefore, all following descriptions and comparisons, except when specified, will be based on the structure of the O monomer.

Conformation of the Thioacyl-GAPDH-NADH Intermediate—The thioacyl-enzyme intermediate is present in each subunit with a refined occupancy factor varying from 0.59 to 0.67. It is well defined with good connectivity in monomers O and R and a real space correlation coefficient of 0.94 (Table 2). In monomers P and Q, density is poorer with a lack of signal at the C2 atom level (Table 2 and supplemental materials). Although no NCS restraints were applied on the bound substrate, the conformation of the intermediate and the interactions shared with the protein structure are almost identical in the four monomers.

The 3-phosphoglyceroyl moiety is bound via a planar thioester bond formed between the sp2 C1 atom and the Sγ atom of the catalytic cysteine (Cys-149) (Fig. 2). The plane of the thioester bond is almost parallel to the one defined by the nicotinamide ring, and the carbonyl oxygen (O1) points in a direction opposite to the catalytic His-176 to accept a H-bond from the Cys-149 main chain and from a water molecule (Fig. 3A). Whereas DL-G3P was used for soaking experiments, the C-2 carbon is found in an R configuration, which accounts for the enantiomer specificity of the GAPDH enzyme toward D-G3P (38). The C2 hydroxyl group points away from the carbonyl group at C1 to adopt a trans conformation. The O2 oxygen atom is assumed to form H-bonds with both the NO7 atom of the cofactor and with the Ne atom of His-176. The C3P group is located in the new Ps site, formed by the 206–212 loop under its B conformation (see below). Its oxygen atoms are hydrogen-bonded to the side chains of Ser-148, Thr-150, and Thr-208 and to the main chain of Thr-150 and Gly-209, all of these residues being invariant. Additional interactions are provided by the non-conserved Arg-195 and by the invariant Arg-231 via two water molecules (see Table 3 for interaction distances).

Given the refined fractional occupancy of this intermediate (Table 2), one has to assume that Cys-149 also exists in its free state. Based on the shape of the density map and on refinements, this residue was built under a unique conformation that is representative of both states. The side chain of the catalytic cysteine superimposes perfectly with that of the known holo-structure from B. stearothermophilus. In its free form, this side chain is well positioned to form an ion pair with the Ne atom of His-176 distant of 3.6 Å.

In each monomer, the cofactor molecule is well defined in density maps with full occupancy. Because the reaction with G3P in the crystal was not complete, a mixture of oxidized and reduced forms is expected for the cofactor. However, the two redox states cannot be distinguished from the 1.77-Å electron density map, and there is no evidence to support any change of conformation for the cofactor molecule after hydride transfer.

A sulfate ion is bound with full occupancy in the Ps site through five hydrogen bonds to Arg-231, Arg-195, and Thr-179 side chains, the 2'-OH atom of the ribose adjacent to the nicotinamide, and a water molecule (Fig. 3A). A second sulfate ion is located in the Ps site with partial occupancy (see above). It interacts with the side chains of Thr-208 and Ser-148 and with the main-chain N atom of Gly-209 (conformation A of the 206–212 loop). This anion exhibits a high B value (45.8 Å²) in comparison with the model mean value (25.7 Å²) and is poorly defined in electron density maps. However, a water molecule cannot account for the observed electron density, and the location of this anion matches the one bound in the Ps site of the holoenzyme structure (10).

Alternate Conformations of the 206–212 Loop—Delineating one side of the active site, the 206–212 loop contributes to the formation of one of the two anion binding sites. As mentioned above, this loop has been unequivocally fitted into the density maps under two conformations (Fig. 4A and Table 2). The first conformation, called conformation A, is strictly equivalent to the conformation described in the holo-structure (10, 16). In this conformation, the loop participates in the binding of a sul-
fate anion in the “classic” P site. The second conformation of
the loop (conformation B), with complementary occupancy,
displays a maximum shift of 3.4 Å for the Gly-209 C/H9251 atom
toward the catalytic cysteine. In this alternate position, the
206–212 loop is located too close to the sulfate molecule to
allow both of them to coexist in the same position, confirming
that the presence of the sulfate anion is associated with the
above described loop conformation A (Fig. 4A). The loop in
conformation B is very similar to the one initially described for
T. maritima GAPDH (see Fig. 4B) (11) and contributes to the forma-
tion of the anion binding site called new P. Both conformations have
nearly identical thermal motion factors (24.5 and 25.2 Å², for confor-
mations A and B, respectively), which fall in the same range of the
overall protein (23.6 Å²), suggesting that both states of the 206–212 loop
are stable conformations.

Comparison with Other Structures—The position of the new P
binding site, here observed for the first time in B. stearothermophilus
GAPDH, is equivalent to that described for structures of GAPDH
from other sources (11–12, 14). Superimposition of the main-chains
atoms of the O subunit of our model with those of the hemithioacetal
structure of E. coli apo-GAPDH (pdb code 1dc4, A chain (14))
locates the two phosphorus atoms only 0.33 Å away from each other
(Fig. 3B). As well, the central atoms of the sulfate and phosphate anions
bound in the new P site of T. mariti-
ma (pdb code 1hdg, O chain (11)) and L. mexicana (pdb code 1gyp, A
chain (12)), respectively, after simi-
lar superimposition procedures, are
situated only 0.17 and 0.42 Å away
from the phosphorus atom of the
thioacylenzyme intermediate, re-
spectively. The position and geo-
metry of the substrate in this inter-
mediate are very close to those
described by Yun et al. (14) for the
hemithioacetal structure (pdb code
1dc4) of E. coli apo-GAPDH. Only a
slight shift can be observed between
the two bound G3P molecules (Fig.
3B) that can be related, first, to the
different hybridizations of the two
C1 atoms, and second, to the steric
influence of the cofactor molecule,
which is missing in the hemithioac-
etal apostructure. As previously
described by Yun et al. (14) for the hemithioacetal intermedi-
ate, the oxygen bound to C1 points away from the hydroxyl
group of C2 to adopt a trans conformation. Thus, the O1 atom
of G3P turns away from the catalytic His-176 in a counterintui-
tive manner with respect to its demonstrated catalytic role (see
“Discussion”).

When the thioacyl-GAPDH structure is compared with the
non-covalent Michaelis complex obtained by Didierjean et al.
(16) for B. stearothermophilus GAPDH (pdb code 1nqo), the

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FIGURE 3. Active site and comparison with other representative complexes. The reaction intermediates, the
substrate and cofactor molecules, and the residues involved in catalysis and substrate binding are presented in stick
mode. Labels 1, 2, and 3 refer to the C1, C2, and C3 atoms of the substrate, respectively. A, stereoscopic view of the
active site of the thioacyl intermediate structure. Hydrogen bonds are presented as dashed lines. Important water
molecules are shown as spheres. B, stereoscopic view of the superimposition between this model (green) and the
hemithioacetal structure of E. coli apo-GAPDH (yellow) (1DC4) (14). C, stereoscopic view of the superimposition
between this model (green) and the Michaelis ternary complex from B. stearothermophilus holo-GAPDH (C1495
mutant, in blue) (1NQO) (16).
main difference concerns the binding of the C3P group, which is located in the P$_s$ site in the non-covalent complex (Fig. 3C), ~6 Å away from the new P$_i$ site. This shift of the C3P group is also accompanied by a complete rearrangement of the carbon chain, which results in significant modifications of its interaction pattern with the protein. Thus, the orientations of the C1-O1 and C2-O2 bonds differ drastically in the two structures. Although the O1 atom points toward the catalytic histidine and the C2-O2 bond is almost perpendicular to the nicotinamide ring in the Michaelis complex, the O1 atom points opposite to His-176, almost parallel to the nicotinamide ring in the Michaelis complex, the O1 atom points away from the histidine and the C2-O2 bond is almost parallel to the nicotinamide ring in the Thioacylenzyme.

**DISCUSSION**

This report presents for the first time the crystal structure of a thioacyl enzyme intermediate of GAPDH. The strategy used to obtain this intermediate takes advantage of the slow rate of the deacylation step in the absence of phosphate ions. Under this condition, the rate of hydrolysis of the intermediate is $10^{-2}$ s$^{-1}$, i.e. $6 \times 10^4$ times slower than acylation (6), which allows the intermediate to accumulate in the crystal. Despite the partial occupancy of the intermediate, the quality of the diffraction data and of the resulting structure allowed us to build two distinct states of the enzyme, which are each present in the four monomers of the enzyme. The most populated state corresponds to the structure of the thioacyl enzyme intermediate covalently bound to the catalytic cysteine and the other one corresponds to a state in which the catalytic cysteine is free and a sulfinate ion is bound in the classic P$_s$ site. Each state is associated with a particular conformation of the 206–212 loop in which invariant Thr-208 and Gly-209 interact with either the C3P of the thioacyl enzyme intermediate or a sulfinate anion.

**Structure of the Thioacyl Enzyme Intermediate and Implications for Catalysis**—The phosphate group of the thioacyl enzyme intermediate is bound in the new P$_i$ site. This result is consistent with the location of the substrate phosphate group found in the structure of the hemithioacetal (sp3) intermediate obtained with the apo-GAPDH from *E. coli*; the two structures superimpose well (Fig. 3B) except at C1 due to different hybridization (sp2 instead of sp3). On the other hand, structures considered to be representative of a productive enzyme-NAD-d-G3P non-covalent complex (16) showed that the P$_i$ site constitutes the binding site for the C3P group of the substrate in this Michaelis complex. Taken altogether, these results suggest that, even in the presence of a fully formed P$_s$ site (presence of the ribose of the cofactor to interact with the P$_s$ site, a condition that was not fulfilled for the hemithioacetal structure from *E. coli* GAPDH in which the enzyme was under its apo-form), the C3P group of the substrate has shifted from P$_s$ site to the new P$_i$ site during the oxidoreduction step. The exact stage at which this shift occurs remains unknown. However, although one cannot rule out the possibility that the formation of the covalent bond between Cys-149 and d-G3P promotes the repositioning of the C3P in the new P$_i$ site, it seems more likely that the first step of the reaction occurs while the C3P group is bound at the P$_i$ site and that the relocation of the phosphate group occurs once hydride transfer is achieved. Indeed, a hydride transfer, while the C3P group is still bound at P$_s$, would present the advantage of holding both the substrate and cofactor in tight interaction and might promote this step of the reaction.

Besides the C3P group location, an unexpected feature concerns the orientation of the oxygen atom bound at C1, which points opposite to His-176, almost parallel to the nicotinamide ring (Fig. 3A), and adopts an energetically favorable trans conformation when related to the O2 atom position. It is well established that an efficient hydride transfer requires either a base-catalyst or an oxyanion hole to stabilize the negative charge developed on the tetrahedral transition state. Whereas non-phosphorylating GAPDHs possess the geometric features of an oxyanion hole reminiscent to those from serine proteases (39), these criteria are clearly not fulfilled in phosphorylating GAPDHs. His-176 was shown instead to play an unequivocal role of base-catalyst during the redox step (5, 7). This means that hydride transfer must have occurred with the C1-OH group well oriented with respect to His-176. In the structure presented here, which is representative of a stage after hydride transfer, the oxygen atom at C1 adopts another position that is well developed on the tetrahedral transition state. Where these two events are coupled or not is still unclear.
However, the fact that both relocations seem to occur at the same stage, that is once hydride transfer is achieved, strongly suggests these two events to be coupled. One should note that, except for the 206–212 loop, the active site conformation remains unchanged between the Michaelis complex and the thioacylenzyme intermediate structures and that only the substrate conformation is modified along the redox step. The pathway leading from one conformation to the other implies mainly a rotation of the trans plane O1–C1–C2–O2 of −180°. A rotation around the C2–C3 bond is also needed for the adequate positioning of the C3P group in the new P site.

The next step consists of the release of the cofactor NADH, which was shown to be rate-limiting in the overall enzymatic process, and the entry of a new molecule of NADH+, which is known to enhance the rate of phosphorolysis (2). The events promoting NADH release are still unclear. However, the loss of the interaction between O2' of the ribose and the C3P group of the intermediate following the flipping of the C3P group from the Ps site to the new Pi site could be the first signal that triggers the dissociation of NADH. Note that, although a mixture of NADH and NADH is present in the structure (due to an incomplete reaction), the two states cannot be distinguished from the electron density maps and have been built as one conformation. Therefore, except for the above mentioned point, no evidence exists in the structure that might account for a destabilization of the cofactor-enzyme complex.

The entry of a new NADH molecule likely promotes conformational changes on the substrate molecule required for phosphorolysis. Indeed, taking into account the facts that (i) His-176 has to play a role as an acid/base catalyst (10) in the phosphorolysis step as it does in the acylation step and (ii) the inorganic phosphate has to bind to either the classic Pi (10) or the new Pi site (12), the consequence of the re-entry of NADH should be a flip back of both the O1 atom and the C3P groups to their initial position to interact with His-176 (Nε) and to bind to the Pi site, respectively. Again, these rearrangements can be coupled or occur in two distinct steps.

**Proposed Scenario**—G3P initially binds to the active site with its C3P located in the Ps site. Reaction begins through the nucleophilic attack of the Cys-149, whose thiol function is first

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**FIGURE 4. Alternate conformations of the 206–212 loop and re-location of the P site anion binding site.**

A, stereoscopic view of the O subunit region spanning residues 205–218. The 206–212 loop is represented in two alternate conformations: in dark gray, the classic conformation delineates the Pi anion binding site where a sulfate is bound (conformation A). In light gray, the alternate conformation exhibits a shift toward the C3P of the intermediate (conformation B). This motion allows Thr-208 and Gly-209 to participate to the formation of the new Pi site and thus to the binding of the C3P group of the intermediate. B, superimposition of the 206–212 loop under its classic conformation (dark gray) with the holo-structure from B. stearothermophilus (in white, pdb code 1gd1 (10)) is reported on the left, and the superimposition of the 206–212 loop with representative GAPDH structures. The superimposition of the 206–212 loop under its alternate conformation with the structure of T. maritima GAPDH (in white, pdb code 1hdg (11)) is reported on the right. All structures are represented in schematic mode with their corresponding bound anion in stick mode. To facilitate comparison, both conformations of our model were represented in each superimposition, shading the one that is not considered.

5 F. Talfournier, unpublished results.
activated through the formation of an ion pair with His-176, on the aldehydic carbon C1 of the substrate (Fig. 5, step 1). Hydride transfer occurs then from the C1 atom of the thiohemiacetal intermediate to the C4 atom of the nicotinamide of NAD\(^+\) through general base catalysis by His-176 (Fig. 5, step 2). At this stage, the C3P group of the thioacyl intermediate is likely still bound in the P\(_s\) site in close interaction with the cofactor (Fig. 5, HTA stage). Then, the thioacylenzyme intermediate undergoes conformational adjustments (Fig. 5, step 3) during which the orientation of the O1 atom changes in such a way that it now points away from His-176 while C3P flips toward the “new” P\(_i\) site. These events lead to the thioacylenzyme intermediate (Fig. 5, TAE stage) whose structure is presented here. The next step consists of the exchange of cofactor (NADH release, entry of a new molecule of NAD\(^+\)) (Fig. 5, step 4). Note that this step is facilitated by the loss of interaction between the substrate and the cofactor molecule due to the relocation of the C3P group in the new P\(_i\) site. The entry of a new NAD\(^+\) molecule likely promotes conformational changes on the substrate molecule that are required for phosphorolysis, that is the flip back of both O1 atom and C3P group to their initial position. Inorganic phosphate finally binds to the active site and attacks the thioacylenzyme intermediate from the P\(_i\) site leading to product (1,3-DPG) release. At the end, the enzyme returns to its holo-form, ready to accommodate a new G3P molecule.

In this scenario, even if the thioacylenzyme structure presented here highlights the structural changes associated with the redox step, further work has to be carried out to understand how this putatively competent complex leads to 1,3-DPG formation. In that context, direct evidence is still lacking as to whether and/or how the redox state of the cofactor promotes the rearrangements required for the phosphorolysis step. In addition, the exact site from which inorganic phosphate attacks the thioacylenzyme is still speculative. The structure of a Michaelis-like complex with 1,3-DPG and NAD\(^+\), and of a thioacylenzyme-NAD\(^+\) complex, will provide further information.
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regarding the mechanism of this “old” but still imperfectly known enzyme.

Acknowledgments—We thank Prof. G. L. Rossi for many stimulating discussions on GAPDH and microspectrophotometry. We gratefully acknowledge access to synchrotron radiation at the EMBL Outstation, Deutsches Elektronen-Synchrotron, Hamburg, and at the European Synchrotron Radiation Facility, Grenoble, France.

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Trapping of the Thioacylglyceraldehyde-3-phosphate Dehydrogenase Intermediate from *Bacillus stearothermophilus*: DIRECT EVIDENCE FOR A FLIP-FLOP MECHANISM

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*J. Biol. Chem.* 2008, 283:21693-21702.
doi: 10.1074/jbc.M802286200 originally published online May 14, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M802286200

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