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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 phosphorolyzing 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.

The atomic coordinates and structure factors (code 3cmc) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/). The abbreviations used are: GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); G3P, D-glyceraldehyde 3-phosphate; 1,3-DPG, 1,3-bisphosphoglycerate; C3P group, C3-phosphate group; TAE, thioacylenzyme; r.m.s.d., root mean square deviation.

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The basis of a model of the thiohemiacetal intermediate in the Hamarus americanus GAPDH structure, the anion binding sites were initially attributed to the specific binding of the C3-phosphate (C3P) group of D-G3P (P, site) and of the inorganic phosphate ion (Pi site). The location of the Pi 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 Pi 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 P, 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
from H. americanus and Bacillus stearothermophilus (9–10), an alternative conformation was first observed in the holoenzyme from Thermostoga maritima and Leishmania mexicana (11–12) that generates a Pᵢ site located closer to the catalytic Cys-149 residue and 3 Å away from the former position, which has been called new Pᵢ site. Whatever the considered position, the Pᵢ site includes the side chains of residues Ser-148 and Thr-208 and the main-chain nitrogen of Gly-209. In addition, the new Pᵢ site also involves residue Thr-150.

Despite numerous functional (6, 7, 13) and structural studies (14–16), the individual role of Pᵢ and Pₛ sites in the catalytic events remains unclear. Although it is generally assumed from geometrical considerations that inorganic phosphate must attack the intermediate from the Pᵢ 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 GAPDH with NAD⁺ and the physiological substrate D-G3P, the phosphate group of which was found to be bound at the Pᵢ site. This result strongly suggests that the Pₛ 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 physiologial 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 thioacyl enzyme 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 Pᵢ 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 Pᵢ site.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—D/L-Glyceraldehyde-3-phosphate (D/L-G3P, Sigma) was prepared from the D/L-G3P diethylacetal 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 × 10⁵ 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 mM 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 suspended at least six times in a stabilizing solution containing 2.7 mM ammonium sulfate, 0.1 mM 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 10× 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 D/L-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 enzyme complex, single crystals were treated with 1 mM sodium arsenate in the presence of G3P. The arsenate anion irreversibly reacts with the thioacyl enzyme, 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 mM ammonium sulfate, 0.1 mM 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
Thioacylenzyme Intermediate from B. stearothermophilus GADPH

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 Dl-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 step 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 \( a = 82 \text{ Å}, b = 123 \text{ Å}, c = 82 \text{ Å}, \) and \( \beta = 111° \), 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 significance and consistent contact with H-bond donor/acceptor. The thioacyl enzyme model obtained from soaked ammonium sulfate-grown crystal was refined up to final convergence using all the reflections (sigma cut-off = 0) with \( R = 0.182 \) and \( R_{\text{free}} = 0.239 \), 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 \( R = 0.161 \) and \( R_{\text{free}} = 0.198 \), 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 thioacyl enzyme 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

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 at 360 nm. Upon addition of G3P to the crystal, a new broad band was observed at around 340 nm, associated with the formation of NADH (35). Upon addition of the physiological substrate G3P to holoenzyme-NAD⁰ 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 NADH transfer from C1 of G3P to C4 of nicotinamide ring and concom-

Thioacyl enzyme intermediate from B. stearothermophilus GADPH

Sulfate (Pi site) Occupancy 0.33 0.47 0.41 0.27

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 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 0.011 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 1gdl) 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 thioacyl enzyme-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

TABLE 1
Data collection and refinement statistics
Values in parentheses refer to the outermost resolution shell.

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

Reﬁnement

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

a R-factor for symmetry-related intensities.

b Crystallographic R-factor.

c Crystallographic R-factor for a randomly selected 5% of reflections not included in refinement.

TABLE 2
Occupancy factor refinement and thermal agitation values

<table>
<thead>
<tr>
<th>Subunit</th>
<th>O</th>
<th>P</th>
<th>Q</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thioacyl intermediate</td>
<td>Refined occupancy</td>
<td>0.64</td>
<td>0.59</td>
<td>0.62</td>
</tr>
<tr>
<td>Mean B value (Å²)</td>
<td>27.3</td>
<td>27.9</td>
<td>26.8</td>
<td>27.6</td>
</tr>
<tr>
<td>Real space correlation coefficient</td>
<td>0.95</td>
<td>0.91</td>
<td>0.94</td>
<td>0.94</td>
</tr>
<tr>
<td>Sulfate (P1 site)</td>
<td>Occupancy</td>
<td>0.53</td>
<td>0.47</td>
<td>0.41</td>
</tr>
<tr>
<td>Mean B value (Å²)</td>
<td>46.1</td>
<td>45.6</td>
<td>45.1</td>
<td>46.3</td>
</tr>
<tr>
<td>206–212 loop (confA/confB)</td>
<td>Refined occupancy</td>
<td>0.47</td>
<td>0.53</td>
<td>0.66</td>
</tr>
<tr>
<td>Mean B value (Å²)</td>
<td>24.8</td>
<td>25.6</td>
<td>24.5</td>
<td>25.2</td>
</tr>
<tr>
<td>Real space correlation coefficient</td>
<td>0.93</td>
<td>0.87</td>
<td>0.95</td>
<td>0.90</td>
</tr>
</tbody>
</table>

a Correlation existing between model density and 2mFo – DFo density.
Thioacylenzyme Intermediate from B. stearothermophilus GADPH

The conformation of the thioacyl intermediate in the GAPDH enzyme is crucial for understanding its function. The thioacyl-GAPDH-NADH intermediate, which includes the thioacyl-enzyme intermediate and the bound substrate, is presented in stick mode in two alternate conformations. The thioacyl-enzyme intermediate is present in each monomer of the O monomer.

The 3-phosphoglyceroyl moiety is bound via a planar thioester bond formed between the sp2 C1 atom and the S of the catalytic cysteine (Cys-149). 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. 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.

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. Although this loop is not directly involved in crystal 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 thioacylenzyme 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. In monomers P and Q, density is poorer with a lack of signal at the C2 atom level (Table 2). In both conformations, the cofactor molecule is well defined in density maps with full occupancy. Given the refined fractional occupancy of this intermediate, the Cys-149 side chain is well positioned to form an ion pair with the N 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 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. 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 Cα 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 formation of the anion binding site called new P. Both conformations have nearly identical thermal motion factors (24.5 and 25.2 Å² for conformations 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. maritima (pdb code 1hdg, O chain (11)) and L. mexicana (pdb code 1gyp, A chain (12)), respectively, after similar superimposition procedures, are situated only 0.17 and 0.42 Å away from the phosphorus atom of the thioacylenzyme intermediate, respectively. The position and geometry of the substrate in this intermediate 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 hemithioacetal apostructure. As previously described by Yun et al. (14) for the hemithioacetal intermediate, 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 counterintuitive 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
main difference concerns the binding of the C3P group, which is located in the Ps site in the non-covalent complex (Fig. 3C), ~6 Å away from the new Pi 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 bound 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 thioacylenzyme.

**DISCUSSION**

This report presents for the first time the crystal structure of a thioacylenzyme 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 thioacylenzyme intermediate covalently bound to the catalytic cysteine and the other one corresponds to a state in which the catalytic cysteine is free and a sulfate ion is bound in the classic Pi 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 intermediate or a sulfate anion.

**Structure of the Thioacylenzyme Intermediate and Implication for Catalysis**—The phosphate group of the thioacyl intermediate is bound in the new Pi site. This result is consistent with the location of the substrate phosphate group found in the structure of the hemithioacetal (sp2) 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 Pi 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 Ps site (presence of the ribose of the cofactor to interact with the Pi 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 Ps to the new Pi site during the oxido-reduction 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 Pi site, it seems more likely that the first step of the reaction occurs while the C3P group is bound at the Ps 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 Ps, 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 results in the disruption of the alignment between the Ne atom of His-176, the O1 and C1 atoms of the substrate, the hydride ion, and the C4 atom of the pyridinium ring.

It appears thus that the overall redox step involves important conformational adjustments of the substrate and requires the relocation of both O1 atom that shifts away from His-176 and of the C3P group that flips from the Pi site toward the new Pi site. Whether 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_i 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 NAD^+, 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 P_i site could be the first signal that triggers the dissociation of NADH. Note that, although a mixture of NAD^+ 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 NAD^+ 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 P_s (10) or the new P_i site (12), the consequence of the re-entry of NAD^+ should be a flip back of both the O1 atom and the C3P groups to their initial position to interact with His-176 (N_e) and to bind to the P_s 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 P_s site. Reaction begins through the nucleophilic attack of the Cys-149, whose thiol function is first

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 NADH 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 Ps 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” Pi 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 NADH) (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 site. The entry of a new NADH 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 Pi site leading to product (1,3-DPG) release. At the end, the enzyme returns to its holoform, 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 thioacyl enzyme intermediate is still speculative. The structure of a Michaelis-like complex with 1,3-DPG and NADH, and of a thioacyl enzyme-NADH complex, will provide further information.

**FIGURE 5. Structural scenario proposed for the catalytic mechanism.** Only hydrogen atoms directly involved in the catalytic mechanism are represented. G3P initially binds to the active site of GAPDH with its C3P group located in the Ps site. Reaction begins through the nucleophilic attack of the Cys-149 thiolate function on the aldehydic carbon C1 of the substrate (step 1), which leads to a tetrahedral intermediate called hemithioacetal (HTA). This step is followed by hydride transfer (step 2), assisted by His-176, from the C1 atom of G3P to the C4 atom of NADH. At this stage, C3P is thought to be located in the Ps site, and the O1 atom of G3P is expected to interact with His-176 (Ne) (see “Discussion”). To allow NADH release, the substrate must undergo a conformational change during which the orientation of the O1 atom changes while the C3P group flips toward the new Pi site (step 3). It results in the loss of the interaction between the substrate and the cofactor required for NADH release (TAE: thioacylenzyme intermediate corresponding to the structure depicted here). NADH enters the active site and likely promotes the relocation of O1 and C3P to their initial position. The Ps site (either in the classic or new position, indicated as “Pi?”) is free to bind an inorganic phosphate for the nucleophilic attack on the thioacyl enzyme. This last step is also assisted by His-176 and leads to the formation of 1,3-bisphosphoglycerate. Subsequent release of the product leaves the enzyme in its holoform, ready to accommodate a new G3P molecule.
Thioacyl enzyme Intermediate from B. stearothermophilus GADPH

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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