Thermal Destabilization of Non-phosphorylating Glyceraldehyde-3-phosphate Dehydrogenase from Streptococcus mutans upon Phosphate Binding in the Active Site

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Catalysis by the NADP-dependent non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN) from *Streptococcus mutans*, a member of the aldehyde dehydrogenase (ALDH) family, relies on a local conformational reorganization of the active site. This rearrangement is promoted by the binding of NADP and is strongly kinetically favored by the formation of the ternary complex enzyme-NADP-substrate. Adiabatic differential scanning calorimetry was used to investigate the effect of ligands on the irreversible thermal denaturation of GAPN. We showed that phosphate binds to GAPN, resulting in the formation of a GAPN-phosphate binary complex characterized by a strongly decreased thermal stability, with a difference of at least 15 °C between the maximum temperatures of the thermal transition peaks. The kinetics of phosphate association and dissociation are slow, allowing both free and GAPN-phosphate complexes to be observed by differential scanning calorimetry and to be separated by native polyacrylamide electrophoresis run in phosphate buffer. Analysis of a set of mutants of GAPN strongly suggests that phosphate is bound to the substrate C-3 phosphate. In addition, the substrate analog glycerol-3-phosphate has similar effects as does phosphate on the thermal behavior of GAPN. Based on the current knowledge on the catalytic mechanism of GAPN and other ALDHs, we propose that ligand-induced thermal destabilization is a mechanism that provides to ALDHs the required flexibility for an efficient catalysis.

Non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN) EC 1.2.1.9) catalyzes the irreversible, NADP-dependent oxidation of D-glyceraldehyde-3-phosphate (D-GAP) into 3-phosphoglycerate. It belongs to the widely distributed aldehyde dehydrogenase (ALDH) superfamily, which participates in many levels of the cellular metabolism in the three kingdoms. As an enzyme involved in the production of NADPH equivalents required in biosynthetic pathways, GAPN is found in photosynthetic and non-photosynthetic cells of higher plants, photosynthetic microorganisms, and some eubacteria, such as certain strains of *Streptococcus* (1, 2). GAPN from *Streptococcus mutans* catalyzes the oxidation of D-GAP via a two-step mechanism, following an ordered sequential mechanism, with NADP acting as the first binding substrate and D-GAP as the second binding substrate. At high concentration, D-GAP behaves as a competitive substrate inhibitor, thus indicating that GAP can bind to the apoenzyme (3). In the acylation step, a covalent thiohemiacetal intermediate is formed with the catalytic residue Cys-302, preceding the oxidoreduction process that leads to a thioacetyl intermediate and formation of NADPH. The deacylation step includes the attack of a water molecule on the thioacetyl intermediate and the release of 3-phosphoglycerate and NADP.

Previous enzymatic and structural studies have highlighted the importance of ligand binding and local reorganization of the active site in the mechanism, affording high catalytic efficiency to GAPN. Specifically, the binding of NADP induces a local conformational rearrangement that leads to the accessibility of the thiol of Cys-302, combined to a shift of $\mu K_{\text{app}}$ from 8.5 to 6.2, and a positioning adapted to subsequently form the competent thiohemiacetal intermediate. This rearrangement of the GAPN-NADP binary complex is strongly kinetically favored when the ternary complex enzyme-cofactor-substrate is formed (4). In addition, the side chains of Glu-268 and Arg-459 rotate, thus leading to the breaking of the ionic bridge that exists in the Apo1 crystal form (see next paragraph). At this point, the side chain of Arg-459 is orientated toward the substrate binding site. During this rearrangement, the distance between the functional groups of Glu-268 and Cys-302 decreases from 8.5 to 3.6 Å. Concomitantly, the oxyanion hole is formed, comprising at least the amide side chain of Asn-169 and the main chain amide of Cys-302, and is postulated to be responsible for the stabilization of the tetrahedral transition states produced during the acylation and deacylation steps (3, 4, 5). In this latter step, the side chain of Glu-268 has been shown to be responsible for the activation of the water molecule involved in the
hydrolysis of the thioacyl intermediate (3). Recent studies on human cytosolic and mitochondrial ALDHs have suggested an isomerization event of the nicotinamide mononucleotide moiety of the cofactor, which would be required for Glu-268 to play this role in the decylation step (6, 7).

Three sulfate binding sites have been described in the crystal structures of the apoforms of GAPN obtained so far, which provided bases for the characterization of the ligand binding sites (5, 8). The first one, the SO₄ site, exists both in Apo1 and Apo2 forms of GAPN and includes Arg-124 and Arg-301 side chains and Arg-459 and Gly-460 main-chain amide groups. In the Apo1 structure, Arg-459 and Glu-268 side chains interact via an ionic bridge, which is split in the Apo2 structure. The second sulfate site, the SO₄b site, has only been found in the Apo2 structure and is shaped by Arg-301 and Arg-459 guanidinium groups, Asn-169 side-chain and Cys-302 and Thr-303 main-chain amide groups. The Thr-303 residue also interacts with the sulfate ions in both sites through the hydroxyl group of its side chain. The sulfate anion bound in the SO₄b site most likely mimics the tetrahedral transition states of the substrate C-1 involved in the acylation and decylation steps. A systematic study of the role of these arginine residues led to the conclusions that Arg-124 is only involved in stabilizing D-GAP and not the catalytic role in the deacylation step (6, 7).

The role of ligand interactions on the structure of the active site, as revealed by enzymatic, biochemical, and structural approaches, raises the question of the identification and characterization of such interactions and of their potential catalytic significance. In this work, we used the thermal unfolding of GAPN followed by adiabatic differential scanning calorimetry (DSC) to investigate the relationships between thermal stability and active site interactions of GAPN with ligands, with respect to the catalytic mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials.** Site-directed Mutagenesis, Production, and Purification of Wild-type and Mutant Enzymes—A DNA plasmid containing the wild-type or mutant cDNA encoding GAPN was carried out as described previously (4). The purification process was carried out in 50 mM potassium phosphate buffer, pH 6.4. Site-directed mutageneses were performed using the method of Kunkel et al. (12) or the QuikChange site-directed mutagenesis kit (Stratagene). The enzymes were isolated as the apoform, judged by the method of absorption ratio A₂₈₀/A₂₆₀ of 2.0 and by the fact that no NADP was enzymatically detectable after acidic denaturation. In the text, GAPN will therefore refer to apoGAPN. Enzyme purity was checked by SDS-PAGE analysis using 7.5% resolving gel. The GAPN monomer concentration was estimated spectrophotometrically at 280 nm, using an extinction coefficient of 5.01 × 10⁴ M⁻¹ cm⁻¹, as deduced by the method of Scopes (13). Subunit molecular mass (~51 kDa) of wild-type and mutant GAPNs were checked by mass spectrometry. For all experiments described, the samples of GAPN were stored in saturated ammonium sulfate in 50 mM potassium phosphate buffer, pH 6.4, at ~20 °C and were desalted by gel filtration, either on a Sephadex G-25 column or an Econo-Pac 10 DG polyacrylamide gel column (Bio-Rad), or dialyzed for at least 20 h against the appropriate buffer before the experiments. Both gel filtration and dialysis procedures gave very similar DSC results. The activity of the enzyme was not significantly affected by the sample preparation.

All other materials were reagent grade or better and were used without further purification. Potassium P₃ was from BDH, NADP was from Roche Applied Science, and DTNB and G3P were from Sigma. DL-GAP (Sigma) was hydrolyzed from DL-GAP diethylacetal according to the manufacturers’ instructions and enzymatically titrated with GAPN.

**Enzyme Kinetics—**Initial rate measurements were carried out at 25 °C spectrophotometrically by following the appearance of NADPH at 340 nm. Because GAPN is equally active with the D and L isomers of GAP, L-GAP was used for activity measurements. The standard experimental conditions were 1 mM NADP, 0.2 mM L-GAP, 50 mM Tes buffer, 5 mM β-mercaptoethanol, pH 8.2. The competitive inhibition constant of G3P for GAP was determined under conditions where GAP does not significantly behave as a competitive substrate inhibitor, i.e. below 0.2 mM (3).

**Differential Scanning Calorimetry—**Differential scanning calorimetry measurements were made using a DASM-4 adiabatic microcalorimeter (Bioprorb, Poushchina, Russia) with 0.47-ml platinum capillary spiral cells at the equilibrium pressure of 2 atmospheres as described previously (14, 15). The calorimetric reversibility of the thermally induced transitions was checked by heating the protein solution in the calorimeter after cooling from the first run, stopped at the end of the transition (i.e. ~55 and ~80 °C for transient B and A, respectively, see “Results”). Temperature scanning rates (β) of 1.5, 1.0, 0.5, and 0.2 °C/min were tested. Subsequently, most experiments in this study were performed at a constant scan rate of 1 °C/min. The thermograms were recorded in the range of 0.1–1.2 mg/ml protein. Instrumental baselines were recorded with both cells filled with buffer were subtracted from the experimental traces to obtain the heat capacity curves (ΔCp, excess heat capacity), versus temperature. Original software was used for recording the DSC data, and MicroCal Origin versions 1.16 and 7.0 were used to determine the Tₛₚ for maximum of the thermal transition peak) and calorimetric enthalpies (ΔHₛₚ) values. For ionic strength controls and kinetic experiments, DSC was performed on a Microcal VP-DSC apparatus equipped with cylindrical 0.54-ml cells under identical conditions, except for the protein concentration fixed at 0.1–0.2 mg/ml. The measurements on the DSC thermograms obtained with both calorimeters under the same conditions were very similar. Because of the irreversibility of the heat denaturation process, it was impossible to calculate the value of the van’ Hoff enthalpy.

**CD Spectroscopy—**Circular dichroism (CD) spectra of GAPN (0.1 mg/ml) were recorded on a Jobin-Yvon CD6 spectropolarimeter using dedicated software. Measurements were recorded in a quartz cuvette with path length of 1, 1.5, and 55 μm. Spectra were recorded as an average of six to eight scans from 205 to 260 nm.

**Gel Filtration—**Gel filtration experiments were carried out on a Superose 12 HR 10/30 column using a Pharmacia fast protein liquid chromatography system at room temperature. Protein (4.9 μM) in a 200-μl aliquot was applied onto the column equilibrated with 50 or 250 mM potassium phosphate buffer, pH 6.2, at 25 °C. The flow rate was 0.5 ml/min, and the protein was detected spectrophotometrically at 280 nm. Thyroglobulin (670 kDa), bovine globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17.5 kDa), and vitamin B₁₂ (1.35 kDa) were used as references to calibrate the column.

**Analytical Ultracentrifugation—**Sedimentation of GAPN was studied using a model E Spinco analytical ultracentrifuge (Beckman-Spinco) in a double-sector cell. A multipurpose analytical ultracentrifuge. An analytical titanium rotor Model F and double-sector cells were used. UV light scanning was carried out at 280 nm. The sedimentation experiments were performed at 25 and 45 °C and 261,600 g for 50 or 250 mM potassium phosphate buffer, pH 8.2. The sedimentation coefficients were normalized to the standard conditions, i.e. a solvent with the density and viscosity of water at 20 °C. Before being used, the cell was filled with buffer and overheating. The sedimentation experiments were performed at 25 °C and 45 °C with water, 261,600 g for 50 or 250 mM potassium phosphate buffer, pH 8.2. The sedimentation coefficients were normalized to the standard conditions, i.e. a solvent with the density and viscosity of water at 20 °C. Before being used, the cell was filled with buffer and overheating. The sedimentation experiments were performed at 25 °C and 45 °C with water, 261,600 g for 50 or 250 mM potassium phosphate buffer, pH 8.2.

**Native PAGE—**Mini Protean 3 cell (Bio-Rad) was used for native PAGE analyses in 8% acrylamide gel (with 2.67% methylene-bis-acrylamide). The temperature of the cell was regulated to 4 °C to avoid overheating. The samples were desalted by gel filtration from the stock suspension in saturated ammonium sulfate into the appropriate running buffer (pH 8.2) prior to the experiment. A voltage of 50 V was used for 1.5 h for gels run in potassium phosphate buffers, pH 8.2, and 100 V for 1 h for gels run in 80 mM Tris-glycine buffer, pH 8.2.

**Inorganic Phosphate Determination—**The C302A GAPN was desalted from the ammonium sulfate stock suspension in 50 mM trisodium phosphate buffer, pH 6.4, by gel filtration in 50 mM ammonium acetate buffer, pH 8.2, or eluted with water from polyacrylamide gel after native PAGE in 80 mM Tris-glycine, pH 8.2, and then precipitated using trifluoroacetic acid before phosphate determination in the supernatant, according to Van Veldhoven et al. and Cogan et al. (16, 17). Briefly, the method is based on the formation of phosphomolybdate complexes revealed by interaction with malachite green under acidic conditions in the presence of polyvinyl alcohol to stabilize the interaction. Ammonio...
FIG. 1. Analysis of the effect of phosphate concentration on the thermal unfolding of wild-type GAPN followed by DSC. The enzyme (0.5 mg/ml) stored as ammonium sulfate precipitate was dialyzed for at least 20 h against potassium Pi buffers, pH 8.2. a, curves 1–8, effect of increasing potassium Pi (KPi) concentration from 4, 12, 25, 50, 125, 250, and 375 to 500 mM, respectively. Components A and B of the thermograms are indicated (see “Results”). b, the reversibility of the effect of phosphate anions. Wild-type GAPN in 500 mM potassium Pi (curve 2) was dialyzed for 20 h against 50 mM potassium Pi buffer, pH 8.2 (curve 1). The thermogram of the enzyme in 50 mM potassium Pi, pH 8.2 (curve 1) is shown for comparison. T, temperature.

nium heptamolybdate in 7.30 M H2SO4, was added at 0.29% (w/v) final concentration, incubated for 10 min at 25 °C, followed by a mixture of malachite green (0.005% (w/v) final) and polyvinyl alcohol (0.05% (w/v) final). After 30 min of incubation at 25 °C, absorbance was measured at 610 nm. At least three independent experiments were performed for each determination.

Accessible Cysteine Content in Enzyme Preparations Measured with DTNB—The amount of reacted cysteines was deduced from the absorbance of thionitrobenzoate released after reaction with DTNB using an extinction coefficient of 13 600 M−1 cm−1 at 412 nm. The enzyme was desalted in 50 mM potassium Pi buffer, pH 8.2, before adding the dehydrasol in 50 mM potassium Pi buffer, pH 8.2, before adding the
development of irreversible thermal unfolding. This precluded a rigorous quantitative analysis of

the DSC data, which were used only to provide estimates of the kinetics of the processes observed, required for this study.

Endotherms A and B Correspond to Two Species of Tetrameric GAPN—Regarding the origin of transitions A and B, the latter could result from the dissociation of the GAPN tetramer into subunits. However, the following experiments excluded this assumption. First, neither the A nor the B transitions were dependent on the protein concentration, indicating that the rate-limiting step of the thermally induced processes occurs prior to the dissociation of the subunits. Second, gel filtration on Superose 12 in the presence of 50 and 250 mM potassium Pi, pH 8.2, showed only one peak corresponding to a molecular weight of $\approx 200,000$ at 25 °C (curves not shown). Third, only one type of species with a sedimentation coefficient ($S_{20,w}$) of 8.32 ± 0.27 S, corresponding to a molecular weight of $\approx 200,000$, was shown from sedimentation experiments run in 50 and 250 mM potassium Pi buffer, pH 8.2, at 25 and 45 °C, respectively. Altogether, these experiments showed that endotherms A and B observed in the presence of phosphate correspond to two species of tetrameric GAPN.

The thermal destabilization of GAPN upon increase of the potassium Pi concentration was further studied by far-UV CD analysis. For GAPN samples containing 10 and 500 mM potassium Pi, pH 8.2, respectively, CD spectra showed similar profiles, thereby indicating that high concentrations of potassium Pi do not significantly affect the secondary structure content of GAPN (Fig. 4, curves 1 and 2). In contrast, after heating at 55 °C for 20 min, drastic changes were observed in the CD profile of the sample containing 500 mM potassium Pi, pH 8.2, as shown by the loss of the content of the secondary structures (Fig. 4, curve 4). The minor changes occurring after thermal treatment of GAPN under the same conditions in the presence of 10 mM potassium Pi, pH 8.2, are thus roughly consistent with the loss of the amount of the thermolabile species existing at this concentration (Fig. 1 and Fig. 4, curve 3).

Finally, two well separated bands were revealed by native PAGE of GAPN solutions run in potassium Pi buffers, pH 8.2 (Fig. 5a). In the presence of increasing concentration of potassium Pi, from 4 to 250 mM, the slower-migrating band became relatively less abundant compared with the faster one. Altogether, this result combined to the DSC and CD data suggests that 1) the slower-migrating band corresponds to the species unfolding in transition A by DSC and 2) phosphate binds to GAPN, resulting in the formation of a GAPN-phosphate binary complex characterized by a lower thermostability (thermal unfolding transition B) and a higher electrophoretic mobility than the unliganded GAPN species.

Phosphate Content in C302A GAPN—To titrate the phosphate content of both GAPN species, the PAGE technique in a buffer devoid of phosphate (i.e. 80 mM Tris-glycine, pH 8.2) appeared as a method of choice to isolate the two forms, provided that the kinetics of phosphate dissociation were slow enough compared with the timescale of the PAGE separation (see “Kinetics of the Phosphate-GAPN Interaction”). Attempts to visualize a band corresponding to the faster-migrating band in samples of wild-type GAPN were unsuccessful. Only one slower-migrating band was seen under these conditions. In contrast, under the same conditions, two bands were visualized for C302A GAPN (Fig. 5b). Therefore, the phosphate content was investigated on each band of C302A GAPN using a colorimetric method based on the interaction of malachite green with phosphomolybdate complexes.

First, the phosphate content in the C302A enzyme sample was determined after desalting from the stock suspension as ammonium sulfate precipitate in 50 mM potassium Pi buffer, pH 6.4, through a Sephadex G-50 column equilibrated with 50 mM ammonium acetate buffer, pH 8.2. Under these conditions, 1 mol of C302A GAPN tetramer contained an average of 1.36 ± 0.09 mol of phosphate. Second, in a separate experiment, the same sample of C302A GAPN was migrated on PAGE run in 80 mM Tris-glycine buffer, pH 8.2. Strips corresponding to each band were cut, and the phosphate content was determined in each band after elution, followed by protein precipitation. Only the strip corresponding to the faster-migrating band contained phosphate, with a stoichiometry of 3.8 ± 0.4 mol/mol of GAPN tetramer, which strongly suggested...
a stoichiometry of 1 phosphate/C302A mutant GAPN subunit. This result, which can reasonably be extrapolated to the phosphate content of the wild-type enzyme, even though the faster-migrating band could not be isolated in the wild type under the conditions used, again supports the existence of a GAPN-phosphate binary complex.

**Kinetics of the GAPN-Phosphate Interaction**—To get insight into the influence of the dynamics of the interaction on the timescale of the DSC and PAGEx experiments, we measured the evolution of the endotherm B area in solutions of wild-type and C302A GAPNs prepared in 500 mM potassium Pi, pH 8.2 (as described under “Experimental Procedures”), dialyzed as a function of time against a 4 mM concentration of the same buffer at 4°C. The data were analyzed as first-order kinetics (curves not shown) to provide estimates for the rate constants of 0.4 ± 0.1 and 0.011 ± 0.003 h⁻¹ (τ½ of 1.7 and 63 h) for wild-type and C302A GAPNs, respectively. Similarly, the kinetics of appearance of the endotherm B were followed by the evolution of endotherm B area in solutions of wild-type GAPN prepared in 4 mM potassium Pi, pH 8.2, dialyzed for increasing time against 500 mM of the same buffer. The rate constant of the process was estimated to 0.3 ± 0.1 h⁻¹ for wild-type GAPN (τ½ of 2.3 h). In addition, when a sample of wild-type GAPN was dialyzed from the stock suspension (in saturated ammonium sulfate in potassium Pi buffer, pH 6.4) against 4 mM potassium Pi buffer, pH 8.2, as a function of time, the DSC analysis revealed that at least a 20-h dialysis was necessary to complete the B→A transformation. Therefore, these conditions were used to prepare GAPN samples in most experiments in this study (see “Experimental Procedures”).

**Analysis of Mutant GAPNs**—As mentioned in the Introduction section, x-ray structures of GAPN showed that three sulfate ions from the crystallization medium can bind to apoGAPN (5, 8). To identify which site bound the phosphate in the GAPN-phosphate binary complex, the thermograms of various mutants of GAPN were recorded under conditions where phosphate concentrations were not saturating. As shown in Fig. 6a, curves 2–6, two endotherms were visualized on the DSC profiles of N169T, T195G, E268A, C302A, and R459I GAPNs in the presence of 50 mM potassium Pi, pH 8.2. Compared with the wild type (Fig. 6a, curve 7), the amount of the thermolabile species was similar in T195G GAPNs, higher in C302A GAPN, but significantly lower in E268A, R459I, and N169T GAPNs. Only the R124L GAPN (Fig. 6a, curve 1) lacked endotherm B at 50 mM potassium Pi, whereas at 500 mM, a faint labile peak appeared. When desalted from saturated ammonium sulfate in 80 mM Tris-glycine buffer, pH 8.2, the R124L, N169T, T195G, E268A, R459I, and the wild-type GAPNs lacked endotherm B (thermograms not shown). In contrast, under the same conditions, the C302A GAPN sample displayed two endotherms (Fig. 6b), in accordance with the results observed in the PAGE experiments (Fig. 5b). Profiles obtained on native PAGE using 50 mM potassium Pi, pH 8.2, as the running buffer were also determined (Fig. 6c). As previously mentioned, two well separated bands were seen under these conditions for wild-type GAPN. Similar to the wild type, N169T and R459I GAPNs exhibited two bands, whereas only one band was visualized for R124L GAPN.

Given the behavior of GAP, which has been shown to bind to apoGAPN, it was interesting to determine the DSC profile of GAPN in the presence of a high concentration of GAP. Unfortunately, under the DSC temperature conditions, GAP is not stable. Consequently, the DSC profile of wild-type GAPN was determined in the presence of 250 mM DL-α-glycerophosphate (G3P), which was shown to behave as a competitive inhibitor toward GAP (Kᵢ = 16 ± 2 mM, data not shown) and had the advantage of being chemically stable under the experimental conditions used. As shown in Fig. 6a, curve 8, two endotherms were visualized at pH 8.2 in 4 mM potassium Pi buffer, pH 8.2, under conditions where no significant thermal transition B is detected by DSC in the absence of G3P (Fig. 1a, curve 1).

**Selectivity of the Anion Binding Site in Wild-type GAPN**—To determine the selectivity of the anion binding site in GAPN, DSC thermograms of solutions of GAPN in 20 mM potassium Pi, pH 8.2, were carried out in the presence of other anions at a fixed concentration of 480 mM. As shown in Fig. 7, in accordance with previous experiments shown in Fig. 2, chloride had no significant effect on the area and $T_{max}$ of endotherm B. In contrast, sulfate induced the increase of the $T_{max}$ and area of endotherm B in a similar manner as phosphate, except that a significant fraction of endotherm A remained. Compared with phosphate 20 mM, the addition of chloride and sulfate resulted in a thermal destabilization of the species unfolding as transition A.

**Characterization of an Inactive Thermostable Species of Wild-type GAPN**—Preliminary experiments, which consisted of
heating GAPN (0.1 mg/ml) at 55 °C in 10 mM potassium Pi, pH 8.2, during 20 min showed that the enzyme had lost its activity, although its content in secondary structures did not significantly change, as shown from CD spectra (Fig. 4). This suggested that, under these heating conditions, the free GAPN was transformed into another form referred to as GAPN*. This was confirmed by experiments that suggested that the GAPN* was not capable of binding phosphate under the same conditions as GAPN. As an example, when a sample of GAPN (1 mg/ml) in 12 mM potassium Pi, pH 8.2 (conditions under which the major species of GAPN is in the non-complexed state) was exposed to a temperature increase from 20 to 55 °C at a constant scan rate of 1 °C/min, followed by cooling to 20 °C and then subjected to a full temperature scan, an endotherm with a T<sub>max</sub> similar to that of endotherm A was revealed, whereas no endotherm corresponding to the thermolabile species was observed (Fig. 8a). Therefore, the endotherm of GAPN* corresponded to a GAPN species that had lost the capability of binding phosphate, even on the timescale of up to 24 h. Native PAGE profile confirmed the DSC analysis of the preheated sample. Only one band, which migrated in a similar manner to that of the band corresponding to the thermolabile species (slow-migrating band), was observed when the native PAGE was run in 12 mM potassium Pi buffer, pH 8.2 (Fig. 8b).

To get more structural information on the different tetrameric GAPN species, the reactivity of cysteines was investigated. In wild-type GAPN, two cysteines (Cys-302 in the active site and Cys-382 located on the surface of the tetramer) are titratable by DTNB (4). In GAPN in 12 mM potassium Pi, pH 8.2, i.e. in conditions under which the main GAPN species is the unliganded one, both cysteines are poorly accessible and thus react slowly (Fig. 8c, curve 1). Under high potassium Pi concentration, in conditions where the major species corresponds to the GAPN-phosphate complex, the cysteines remained poorly accessible (data not shown). By contrast, as shown in Fig. 8c, when wild-type GAPN (1 mg/ml) in 12 mM potassium Pi buffer, pH 8.2, was heated at 55 °C during 20 min, both cysteines reacted rapidly with DTNB (Fig. 8c, curve 2), suggesting that the accessibilities of Cys-302 and Cys-382 strongly increase in the GAPN* species.

**DISCUSSION**

**Evidence in Favor of the Existence of a Phosphate Binding Equilibrium on GAPN—**The DSC analysis of solutions of GAPN in potassium Pi buffers, pH 8.2, showed the presence of two thermally induced transitions A and B of GAPN, characterized by high and low T<sub>max</sub>, respectively. From native PAGE, gel filtration, and sedimentation experiments, endotherms A and B were assigned to two species of tetrameric GAPN, both unfolding irreversibly on temperature increase. The species corresponding to endotherm B would differ from that of endotherm A by a lower thermostability and a higher electrophoretic mobility due to the presence of 4 mol of phosphate/mol of tetramer. Therefore, endotherm A would represent the thermal unfolding of free GAPN, and endotherm B, the thermal unfolding of the GAPN-phosphate binary complex. This result, obtained by direct phosphate titration on both individual species of the C302A GAPN isolated by native PAGE, was further supported by the dependence of the T<sub>max</sub> of the thermolabile species on phosphate concentration for the wild-type enzyme, which increases linearly as a function of ln[phosphate] (not shown). Such a behavior suggests that phosphate likely dissociates prior to the rate-determining step of the thermal unfolding process (19), thus implying that this species corresponds to a complex of GAPN with phosphate. For thermostable A species, on the contrary, the T<sub>max</sub> values decrease for increasing phosphate concentrations, whereas the line shape of the corresponding peak flattens. Furthermore, the effect of high concent-
trations of NaCl on endotherm A in the presence of 20 or 50 mM phosphate is similar to the effect of phosphate at similar ionic strength (Fig. 2). Because these effects are significant only at high concentrations, they are consistent with a thermal destabilization effect due to either an increase of the ionic strength or to a nonspecific binding of phosphate on GAPN.

Analysis of the titration of GAPN by phosphate using DSC showed the increase and decrease of the areas of endotherms B and A, respectively. This behavior suggests that, in the presence of phosphate, before heating, a binding equilibrium exists between GAPN and phosphate. However, as a consequence of the irreversible nature of the mechanisms of thermal denaturation, the \( \Delta H_{\text{cal}} \) values could be affected by effects such as aggregation or phosphate dissociation in addition to the concentration of the B form by itself. Therefore, the data reported in Fig. 1 could not be rigorously exploited to determine a dissociation constant for phosphate. The hypothesis of the existence of a binding equilibrium was further supported by the reversibility of the phenomenon when a GAPN solution in 500 mM potassium Pi was extensively dialyzed against the same buffer at 50 mM. However, the timescales of the experimental approaches that allowed us to identify the free and liganded species of GAPN preclude the observation of relatively fast equilibria. Indeed, assuming the existence of a fast equilibrium, the thermal denaturation of the GAPN-phosphate (thermolabile) species during the DSC experiment should drive the equilibrium toward the binding of phosphate, preventing the observation of endotherm A (free GAPN). Similarly, the separation of both forms by PAGE should result in a continuous smear between the bands. Yet, both species were resolved by DSC and PAGE, indicating the possibility of a slower equilibrium. The kinetics of the increase of the area of endotherm B for wild-type GAPN in the presence of 500 mM potassium Pi were characterized as a first-order process with a half-time estimated to be \( \approx 2 \) h. Therefore, because the thermal denaturation of both species by DSC was, at most, 30 min apart (for a scan rate of 1 °C/min), a maximum of 15% of free GAPN could potentially be transformed into the liganded form in the course of the experiment. In addition, heating to 55 °C in the DSC apparatus resulted in the transformation of free GAPN into GAPN*, which appeared to be irreversible on the timescale of the experiment, thereby trapping the protein as GAPN* during the thermal scanning (this might also be true during the PAGE separation). However, this transformation could, by itself, drive the equilibrium toward the dissociation of phosphate. Because the kinetics of the decrease of the area of endotherm B in a solution dialyzed from 500 to 4 mM potassium Pi are slow on the timescale of the DSC experiment (half-time of 1.8 h), the endotherm B was expected not to be perturbed by the GAPN→GAPN* process along the DSC experiment. Besides, the kinetics of the thermal unfolding, by themselves, could interfere with the analysis of the equilibrium. However, the timescale of the denaturation, with rates that depend on the temperature and fall into the 3–100-h \(^{–1}\) range, from the beginning to the end of the transition peak (according to the analysis as a simple two-state kinetic model), are fast compared with the kinetics of evolution of the area of endotherm B. Consequently, the DSC analysis can almost be considered as a snapshot of the composition in free and phosphate-complexed populations of GAPN in solution. The same rationale likely also applies to the PAGE experiments that are performed on a similar timescale as DSC (\(<2\) h).

On these bases, several lines of evidence argue in favor of an equilibrium of association/dissociation of phosphate to GAPN. First is the fact that the formation of the GAPN-phosphate binary complex specifically depends on the phosphate concentration and not on ionic strength; therefore, the saturation of the dependence of the area of endotherm B on the phosphate concentration, even if it cannot be rigorously exploited to determine a dissociation constant, is another indicator of the existence of an equilibrium. Second is the reversibility of the conversion between both species upon dialysis against lower phosphate concentration and, in addition, the fact that these processes could be followed kinetically in both directions. Third is the demonstration that the C302A mutant of GAPN binds phosphate ions with a defined stoichiometry of 4 mol of phosphate ion/tetramer by direct phosphate determination, a result that can reasonably be extrapolated to the wild type. Such conclusion is also supported by the separation of both species by PAGE, showing a higher electrophoretic mobility for the GAPN-phosphate complex, consistent with the binding of the negatively charged phosphate ion to GAPN and by the dependence of the \( T_{\text{max}} \) of endotherm B on phosphate concentration.

If other anions than phosphate were able to bind to GAPN, they could thus be compared with phosphate by the DSC technique. As shown in Fig. 7, sulfate can bind to GAPN, contrary to chloride. Qualitatively, from the \( \Delta H_{\text{cal}}(B) \) values, phosphate appears to bind more specifically to GAPN than sulfate. This result again supports the existence of a specific interaction between phosphate and GAPN, because a specific effect is obtained with an ion of similar geometry, i.e. sulfate.

The comparison between the kinetics of phosphate dissociation for the wild-type and C302A GAPNs shows that it occurs \(-30\)-fold slower for the mutant. This is probably the reason why the characterization of both species of the C302A GAPN by PAGE was possible in the absence of phosphate. Furthermore, the presence of phosphate in the isolated faster-migrating band of C302A GAPN in these conditions suggests a strong affinity of this mutant for phosphate relative to sulfate, because phosphate remains bound even in the presence of saturated ammonium sulfate concentration in the storage suspension.

**Structural Aspects of the Binding**—The study of a set of GAPN mutants showed that, only in the thermogram of the R124L GAPN, the peak corresponding to the complex is absent and, only in the native PAGE analysis of this mutant, no significant trace of a band corresponding to the complex is revealed. Because the Arg-124 residue is only involved in the binding of the phosphate group of GAP (9), these observations strongly suggest that it represents the specific site of interaction of phosphate on GAPN. However, this assignment does not provide a clear explanation for the higher apparent affinity of phosphate for C302A GAPN compared with the wild type, although the removal of the negative charge of the Cys-302 thiolate in the mutant could relieve a possible repulsion with the phosphate bound.

If phosphate binds to the C-3 subsite of the GAP binding site, then other specific ligands should be able to bind to this subsite. Previous study shows that GAP behaved as a competitive substrate inhibitor with a \( K_I \) of 0.54 mM (3). This indicated that, at high concentration, GAP binds to the apoenzyme, causes a conformational change that closes the active site, and thus prevents NADP from binding. This hypothesis was tested with G3P, a competitive inhibitor of GAP. As shown in Fig. 6a, at low potassium Pi concentration, in which the phosphate-complexed population is not significant, the addition of G3P resulted in the emergence of endotherm B, supporting the binding of this analog to the same site as phosphate and as the C-3 phosphate of GAP.

**Relevance to the Enzymatic Mechanism**—This study describes a very unusual, strong thermal destabilization of GAPN upon phosphate binding. Several arguments support the idea that the above described phenomenon is relevant to the catalytic mechanism. First, phosphate specifically binds into a de-
fined subsite of the substrate binding pocket. Second, the substrate analog G3P also binds to GAPN and generates a GAPN complex with similar thermal behavior as the phosphate complex. Third, a comparable destabilizing effect is observed when NADP (but not NAD) binds to GAPN. Indeed, the addition of increasing concentrations of NADP results in the emergence of NADP (but not NAD) binds to GAPN.3 Indeed, the addition of a comparable destabilizing effect is observed when GAPN ternary complex have convincingly demonstrated the occurrence of the isomerization conformational step as a flip movement of the nicotinamide moiety of the coenzyme likely occurs during the catalytic cycle. This movement of the cofactor would be a prerequisite for Glu-268 to be in a competent position to act as a base in the hydrolysis of the thioacylenzyme intermediate (6, 7). In addition, recent structural x-ray data on a GAPN ternary complex have convincingly demonstrated the occurrence of the isomerization conformational step as a flip movement of the nicotinamide moiety of the cofactor, strongly suggesting a link between this cofactor flip rearrangement, conformational flexibility of the active site, and thermal destabilization occurring upon substrate and/or cofactor binding. Therefore, on the basis of the present work and of the various studies on different ALDHs, we propose that ligand-induced thermal destabilization is a mechanism that provides to ALDHs the required flexibility for an efficient catalysis.

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Thermal Destabilization of Non-phosphorylating Glyceraldehyde-3-phosphate Dehydrogenase from *Streptococcus mutans* upon Phosphate Binding in the Active Site

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