Characterization of the Amino Acids Involved in Substrate Specificity of Nonphosphorylating Glyceraldehyde-3-Phosphate Dehydrogenase from Streptococcus mutans*

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In order to address the molecular basis of the specificity of aldehyde dehydrogenase for aldehyde substrates, enzymatic characterization of the glyceraldehyde 3-phosphate (G3P) binding site of non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN) from Streptococcus mutans has been undertaken. In this work, residues Arg-124, Tyr-170, Arg-301, and Arg-459 were changed by site-directed mutagenesis and the catalytic properties of GAPN mutants investigated. Changing Tyr-170 into phenylalanine induces no major effect on $k_{cat}$ and $K_m$ for D-G3P in both acylation and deacylation steps. Substitutions of Arg-124 and Arg-301 by leucine and Arg-459 by isoleucine led to distinct effects on $K_m$, on $k_{cat}$, or on both. The rate-limiting step of the R124L GAPN remains deacylation. Pre-steady-state analysis and substrate isotope measurements show that hydrate transfer remains rate-determining in acylation. Only the apparent affinity for D-G3P is decreased in both acylation and deacylation steps. Substitution of Arg-459 by isoleucine leads to a drastic effect on the catalytic efficiency by a factor of 103. With this R459L GAPN, the rate-limiting step is prior to hydrate transfer, and the $K_m$ for D-G3P is increased by at least 2 orders of magnitude. Binding of NADP leads to a time-dependent formation of a charge transfer transition at 333 nm between the pyridinium ring of NADP and the thiolate of Cys-302, which is not observed with the holo-wild type. Accessibility of Cys-302 is shown to be strongly decreased within the holostructure. The substitution of Arg-301 by leucine leads to an even more drastic effect with a change of the rate-limiting step similar to that observed for R459I GAPN. Taking into account the three-dimensional structure of GAPN from S. mutans and the data of the present study, it is proposed that 1) Tyr-170 is not essential for the catalytic event, 2) Arg-124 is only involved in stabilizing D-G3P binding via an interaction with the C-3 phosphate, and 3) Arg-301 and Arg-459 participate not only in D-G3P binding via interaction with C-3 phosphate but also in positioning efficiently D-G3P relative to Cys-302 within the ternary complex GAPN·NADP·D-G3P.

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Nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN) catalyzes the irreversible oxidation of D-glyceraldehyde-3-phosphate (D-G3P) into 3-phosphoglycerate in the presence of NADP via a two-step chemical mechanism. It belongs to the aldehyde dehydrogenase (ALDH) superfamily, which oxidizes a wide variety of aldehydes into nonactivated or SCoA-activated acidic components (1–3). These enzymes play parts at several levels of cellular metabolism including anaerobic and catabolic pathways, detoxification processes, and embryogenesis development. Their numerous functions probably explain why most ALDHs show a wide substrate specificity, except for the CoA-dependent ALDH. In mammals, ALDH class 1 shows preference for retinal but it can also oxidize aromatic and “long-chain” aldehydes with dissimilar catalytic efficiencies, whereas ALDHs that belong to class 2 are more specific for acetaldehyde and short-chain aldehydes (4, 5). Inspection of the active site of the ALDH structures (i.e. rat dimeric class 3 ALDH, bovine mitochondrial ALDH, sheep liver cytosolic ALDH, rat retinal dehydrogenase type II, cod liver betaine ALDH, human liver mitochondrial ALDH, ALDH from Vibrio harveyi, and GAPN from Thermoproteus tenax (6–13)) gives no clear information on the binding mode of the aldehyde substrate. Therefore, characterizing the molecular and structural factors involved in substrate specificity is important for a better understanding of the efficiency of the catalytic event and of the evolution of the active sites of ALDHs.

Unlike most ALDHs, GAPN is an ALDH for which the physiological substrate is known. The fact that GAPN shows high catalytic efficiency toward D-G3P implies prerequisites with respect to the chemical mechanism. Previous enzymatic and structural studies of GAPN from Streptococcus mutans have shown that NADP binding induces a local conformational rearrangement. As a consequence, the thiol of Cys-302 becomes accessible, its $pK_{app}$ shifts from 8.5 to 6.2, and it is now well positioned to subsequently form a competent thiohemiacetal intermediate. On the other hand, the side chains of Glu-288 and Arg-459 also rotate and then do not interact with each other anymore. The side chain of Arg-459 is now oriented toward the substrate binding site. Glu-288 now has a $pK_{app}$ that shifts from probably 5.7 in the apo structure to 7.6 in the holo form and has its side chain well positioned for subsequent activating the water molecule involved in hydrolysis of the thioacyl intermediate. During this rearrangement, the distance between Glu-288 and Cys-302 passes from 7.6 to 3.6 Å. Concomitantly, the oxi洋洋on hole, composed of at least the...
amide side chain of Asn-169 and the NH main chain of Cys-302, is formed. Its role is to stabilize the tetrahedral transition states formed during acylation and deacylation steps. This local conformational change has been shown to be strongly favored by binding of D-G3P to the binary complex GAPN-NADP (14–16). Whereas the rate-limiting step of GAPN is deacylation, that of human liver mitochondrial ALDH has been shown to depend on the chemical structure of the aldehyde substrate (17). Thus, it appears that the nature of the substrate can modulate the formation of a competent ternary complex.

This justifies the determination of the structural factors that are implied in the catalytic efficiency of GAPNs. Several crystal structures of GAPN from S. mutans have been described so far (15, 18). The apo1 structure shows a sulfate anion, called SO4a, but via its NH main chain. The apo2 structure shows groups. Arg-459, which is located within a loop, also interacts loop, respectively, interact with SO4a via their guanidinium hydroxyl group, which in turn also interacts with SO4a.

A second sulfate anion, called SO4b, which probably mimics the spatial position of the C-3 phosphate of G3P. Arg-124 and Arg-301, which are located in an -helix and within a loop, seem to be specific to GAPN proteins. Here, Arg-124 and Arg-301 are substituted by leucine, and Arg-459 and Tyr-170 are changed into isoleucine and phenylalanine, respectively. The catalytic properties of these GAPN mutants were determined. Altogether, the results suggest that Tyr-170 has no significant role. Arg-124 participates only in D-G3P binding via interaction with C-3 phosphate, and both Arg-301 and Arg-459 are not only involved in D-G3P binding but also participate in positioning efficiently the substrate with respect to Cys-302 within the ternary complex GAPN-NADP-D-G3P.

**EXPERIMENTAL PROCEDURES**

Materials—Production and purification procedures of wild-type and mutated GAPNs were carried out as previously described (14). GAPN concentration was determined spectrophotometrically as the apo form by using a molar extinction coefficient of 2.04 × 10^4 M^-1 cm^-1 at 280 nm. Enzyme concentrations were expressed per monomer (normality, N). All other materials were reagent grade or better and were used without further purification. NADP (Roche Molecular Biochemicals) and 2,2'-dithiopyridyldisulfide (2PDS) were dissolved in H₂O, and the stock concentrations were determined spectrophotometrically by using a molar extinction coefficient of 18,000 M^-1 cm^-1 at 260 nm and 10,100 M^-1 cm^-1 at 254 nm, respectively. D-G3P (Sigma) was hydrolyzed from D-G3P diethyl acetal according to the manufacturers’ instructions and enzymatically titrated with GAPN.

Enzyme Kinetics and Determination of K_m Values for NADP and D-G3P Constants—To determine the nature of the rate-limiting step, transient kinetics of GAPN mutants were carried out at pH 8.5 with a mixed buffer (buffer A) containing 30 mM acetic acid, 30 mM imidazole, and 120 mM Tris/HCl at constant ionic strength of 0.15 M, as already described by Marchal et al. (16). Fast kinetic measurements were carried out on a SPM 3 Biologic Instruments stopped-flow apparatus (Biologic, Grenoble, France) with a tungsten lamp and slit of 5-nm light source equipped with a 1-cm light path TC100-10 cuvette maintained at 25 °C by a circulating water bath. One syringe was filled with mutant enzymes (16 μM after mixing), and the other one contained NADP and D-G3P (1 mM after mixing). Under these conditions, the acylation step was shown to be rate-limiting for only the R301L and R459I mutant GAPN proteins (see “Results”). Kinetic analyses of the acylation step were carried out on a Kontron Uvikon 935 spectrophotometer by following the appearance of NADPH at 340 nm for both GAPN mutants. For the determination of kinetic parameters, initial rate measurements were carried out under the following conditions: 50 mM TES/NaOH buffer, pH 8.5, and 5 mM β-mercaptoethanol (ionic strength 0.05 M).
G3P Recognition Subsite of GAPN

using the following range: 0.05–4 mM n-G3P (with 1 mM NADP), 0.01–1 mM NADP (with 4 mM n-G3P). 0.25 mM (NH4)2SO4 was added in the reaction mixture for the R459I GAPN. \(^2\) Data were collected in triplicate, and kinetic constants were determined by Michaelis-Menten analysis. The acylation rate constant \(k_{ac}\) was studied over a pH range of 5–9 with the buffer A. 0.25 mM (NH4)2SO4 was added in the reaction mixture for the R459I GAPN protein. The data were expressed as initial rate constants per mg protein. Initial activity measurements were carried out at subsaturating concentrations of n-G3P (see "Results"). The second-order rate constant \(k_{cat}/K_m\), which corresponds to the efficiency of the acylation step, was calculated at each pH, dividing \(k_{ac}\) by the concentration of n-G3P and was fitted to Equation 1 or 2 to determine the best-fit \(pK_a\) values, where \(y_{max}/K_m\) represents the maximum pH-independent second-order rate constant,

\[
\frac{k_{cat}}{K_m} = \left(\frac{k_{cat}}{K_m}\right)^{max} \left(1 + \frac{1}{1 + 10^{pK_a - pH}}\right) \quad \text{(Eq. 1)}
\]

Where \(k_{cat}/K_m\) and \(k_{cat}/K_m^{max}\) represent the characteristic pH-independent second-order rate constants and \(pK_a\) and \(pK_a\) reflect the different groups involved in the acylation step (16). In the present work, an error structure of constant relative error was assumed, and weighting factors were inversely proportional to \(k_{cat}/K_m^{max}\).

In a previous study, it was shown that acylation and deacylation steps of wild-type GAPN could be kinetically resolved and that deacylation was rate-limiting under steady-state conditions (16). It was also demonstrated that the rate-limiting step in deacylation was associated with hydrolysis, whereas hydride transfer was rate-determining in acylation.

\[e = e_{cat} + \frac{e_a - e_{cat}}{1 + 10^{pK_a - pH}}\]  
\[k_{diss} = k_d\left(\frac{1}{10^{pK_a - pH} + 1}\right) + k_a\left(\frac{1}{10^{pK_a - pH} + 1}\right)\]  

RESULTS

Kinetic Properties of R124L, Y170F, R301L, and R459I GAPNs—In a previous study, it was shown that acylation and deacylation steps of wild-type GAPN could be kinetically resolved and that deacylation was rate-limiting under steady-state conditions (16). It was also demonstrated that the rate-limiting step in deacylation was associated with hydrolysis, whereas hydride transfer was rate-determining in acylation.

\[k_{cat} = k_d\left(\frac{1}{10^{pK_a - pH} + 1}\right) + k_a\left(\frac{1}{10^{pK_a - pH} + 1}\right)\]  

When cysteine reactivities were low, reactions were carried out on a Kontron Uvikon 933 spectrophotometer. The pH dependences of the 2PDS reactions with NADP-bound apo \(^2\) or apo-R301L and holo-R459I GAPN mutants were performed at 25 °C in buffer A over the pH range of 5.0–9.5 under pseudo-first-order reaction conditions. The pseudo-first-order rate constants \(k_{cat}\) and \(k_a\) were determined at each pH by fitting the absorbance A at 343 nm versus time t to Equation 4 for a double exponential profile, where \(a_1\) and \(a_2\) represent the burst magnitudes corresponding to each thiolate species, \(k_1 = k_{cat}\) and \(k_2 = k_{diss}\), and \(c\) represents the value of the ordinate intercept.

\[A = a_1(1 - e^{-k_1t}) + a_2(1 - e^{-k_2t}) + c\]  

The second-order kinetic constants \(k_{cat}\) were calculated dividing the \(k_{diss}\) values by the concentration of 2PDS and were fitted to Equation 3 to determine the best-fit \(pK_{app}\) values.

Measurements of the absorbance of NADP-thiolate interaction within mutant GAPN R459I were recorded in a 1.0-cm path length cuvette in a Uvikon 933 spectrophotometer at 25 °C. The R459I mutant samples were diluted in buffer A with 0.25 mM (NH4)2SO4 at a final concentration of 20 mM and were incubated 5–30 min in the presence of 1 mM NADP. Data acquisition was in steps of 1 nm from 395 to 290 nm over a pH range of 5.5–9.0. The protein solution was scanned relative to the buffer solution containing 1 mM NADP. The differences were then converted to molar absorption coefficients at 333 nm, and data were fitted to a model derived from the Henderson-Hasselbalch equation for one \(pK_{app}\),

\[pH = pK_{app} + \log_{10}\left(\frac{[H^+]^2}{2[H^+][OH^-]}\right)\]

where \(k_{cat}\) and \(k_{diss}\) are the molar absorption coefficients of the thiol and thiolate species in the binary complex of enzyme-NADP.

Time courses of formation of NADP-thiolate interaction in R459I GAPN were followed by the appearance of the absorption band at 333 nm, at a constant temperature of 25 °C, over the pH range of 5.5–9.5. At each pH, the enzyme (20 mM final concentration) was dissolved in the buffer A with 0.25 mM (NH4)2SO4, 1 mM NADP was added, and the absorbance was recorded at 333 nm. Pseudo-first-order rate constants \(k_{diss}\) were determined at each pH by fitting the absorbance A at 333 nm versus time t to an equation for a single exponential profile and were fitted to Equation 6 to determine the best-fit \(pK_{app}\) values, where \(k_1\) and \(k_2\) represent two different rate constants for the thiolate form.

\[k_{diss} = k_d\left(\frac{1}{10^{pK_a - pH} + 1}\right) + k_a\left(\frac{1}{10^{pK_a - pH} + 1}\right)\]

\(^2\) Usually, wild-type GAPN remains stable at least 3 days after dialysis against a 50 mM phosphate buffer, pH 6.4, at 4 °C. Under similar conditions, R459I mutant loses its activity. Only the addition of ammonium sulfate (NH4)2SO4 (0.25 mM final concentration) in the phosphate buffer permits storage of the mutant enzyme without loss of activity. For the kinetic assays, (NH4)2SO4 was systematically added at a concentration of 0.25 mM.
Kinetic parameters and nature of the rate-limiting step for mutant GAPNs

| D-G3P substrate | K_m (mM) | k (s⁻¹) | Limiting step |
|-----------------|---------|---------|---------------|
| Wild-type GAPN  | 0.046 ± 0.001 | 0.54 ± 0.02 | 60 ± 1 | Deacylation |
| R124L mutant   | >15     | 2.5 ± 0.1 | 103 ± 2 | Deacylation |
| R301L mutant   | >15     | 0.060 ± 0.007 | 0.28 ± 0.02 | Acylation |
| R459I mutant   | 9.2 ± 0.9 | 1.9 ± 0.2 | Acylation |

* For the R124L and R301L mutants, only an estimation of the K_m value for D-G3P could be given. The observed rate constants k were determined at a concentration of 4 mM D-G3P.

* The nature of the rate-determining step was deduced from pre-steady-state kinetic experiments. (see “Results” for further details).

The situation is different for the Y170F mutant GAPN protein. Under steady-state conditions, the K_m value for D-G3P was only increased by a factor of 4, whereas the k_cat constant was even higher compared with wild type (Table I). The K_m and k_cat values for D-G3P were also determined under pre-steady-state conditions at pH 8.5 and at 10 °C. They were shown to be 0.67 mM and 260 s⁻¹, respectively. Compared with k_cat/K_m values for D-G3P, these values are similar to those determined for the wild type.

R301L and R459I GAPNs—For the R301L mutant, no saturating K_m effect for D-G3P was observed in the acylation step up to a concentration of 2 mM. Only an estimation of the K_m value could be obtained (i.e. higher than 15 mM). Also, the substitution of Arg-459 by isoleucine induced a large increase of the K_m value for D-G3P up to 9.2 mM (Table I). These values are at least 20-fold higher than that of wild type.

No substrate kinetic isotope effect was observed with D-[1-²H]G3P for either GAPN mutants (Table II). Thus, the rate-determining step for both mutants is associated with a step prior to hydride transfer (Table II). The second-order rate constant k_cat/K_m was decreased by a factor around 10³ relative to wild type. Taking into account the K_m values, this indicates that the rate of the new limiting step is at least 2500-fold less efficient than with the wild type.

The low catalytic efficiency of the acylation step for both mutants is probably the consequence of the formation of an inefficient ternary complex GAPN:NADP:D-G3P due to either an unsuitable positioning of the aldehyde of D-G3P with respect to Cys-302 or to a low chemical reactivity of Cys-302 or both. A low chemical reactivity could be due to either a high pK_a of Cys-302 or a low accessibility of the thiolate of Cys-302 or both. To explore these different possibilities, two approaches were used. In the first one, the rate of acylation was studied over a pH range of 5–9. k_cat/K_m values for R459I mutant GAPN protein were determined at subsaturating concentration of 1 mM of D-G3P under conditions where D-G3P does not behave as a competitive inhibitor. As shown in Fig. 2, the pH dependence of k_cat/K_m exhibited a double-sigmoidal profile with pK_a of 6.7 and 7.6. Compared with wild type, pK_a of 6.7 would correspond to that of Cys-302, whereas the one at 7.6 would be that of Glu-268. Another means to determine pK_a of cysteine is to use a specific kinetic probe like 2PDS. This has the advantage of also probing thiolate accessibility. This allowed us to show that (i) Cys-302 and Cys-382 in apo wild type have similar pK_a values around 8.5–9.0 and exhibit a low reactivity with k_cat/K_m values of 225 M⁻¹s⁻¹ and 475 M⁻¹s⁻¹, respectively, which indicates a low accessibility of both cysteines; (ii) Cys-302 is accessible within the holo form as shown by a k_cat value higher than 2 × 10⁷ M⁻¹s⁻¹; and (iii) Cys-382, which...
**TABLE II**

Parameters for the pH dependence of the acylation second rate constant for the mutant GAPNs

| K_m (mM) | pK_{a1} | pK_{a2} | k_{app}/K_m (M⁻¹s⁻¹) | Substrate isotope effect |
|----------|---------|---------|----------------------|------------------------|
| Wild-type GAPN | 0.49 ± 0.05 | 6.2 ± 0.1 | 7.5 ± 0.1 | 1.0 × 10⁶ | 2.7 |
| R124L mutant | 88 | 6.1 ± 0.1 | 7.3 ± 0.2 | 2.1 × 10⁵ | 2.1 |
| R301L mutant | >15 | ND² | ND | 15⁴ | 1.2 |
| R459I mutant | 9.2 ± 0.9 | 6.7 ± 0.1 | 7.6 ± 0.2 | 50⁴ | 1.3 |

¹ From Ref. 16. The k_{app}/K_m value for the wild type was calculated from the acylation rate constant determined at 10 °C and at 0.2 mM D-G3P (16) (see Footnote 4).

² The K_m value for D-G3P was estimated from double reciprocal plots of the acylation rate constant k_{app} versus D-G3P concentrations (0.5–9 mM).

³ From Table 1.

⁴ ND, not determined.

⁵ For the R301L and R459I mutants, the second-order rate constants k_{app}/K_m were calculated by dividing the observed rate constant by the concentration of 4 mM and 1 mM D-G3P, respectively. The values indicated are those obtained at optimum pH of 8.5. The substrate isotope effects were calculated as described under “Experimental Procedures.”

is located at the surface of the tridimensional structure, has a pK_{app} value within the holo form that remains the same as in the apo form and displays a low accessibility as shown by a k_{app} value of 13 M⁻¹s⁻¹ (14). For the R459I mutant of GAPN apo-enzyme, two cysteines were reactive with 2PDS but with distinct k_{app} constants (data not shown). The pH-k_{app} curve for each thiolate form exhibited a monosigmoidal profile with a pK_{app} value of 8.5 and higher than 9.2 and k_{app} values of 18 × 10⁴ M⁻¹s⁻¹ and 6 × 10⁴ M⁻¹s⁻¹ at pH 9.5, respectively (see Fig. 3, solid symbols). From the comparison of the data obtained for the titrations of Cys-302 and Cys-382 by 2PDS in the C382A and C302A GAPN mutants, respectively (14), the pK_{app} of 8.5 and of 9.2 could be assigned to those of Cys-302 and Cys-382, respectively. The fact that the Cys-302 has a pK_{app} value similar to that of Cys-302 in wild-type apoenzyme but with a high k_{app} value of 18 × 10⁴ M⁻¹s⁻¹ indicates that Cys-302 is accessible within the active site of R459I GAPN. This result remains to be explained but is reminiscent a similar situation encountered with other active site mutants such as the N169A mutant GAPN protein. Clearly, the energetic barrier between both Cys-302 conformers should be small, and therefore the proportion of both populations is likely to be controlled by a kinetic effect. The high accessibility of Cys-302 in R459I GAPN is probably not due to the presence of 0.25 M (NH₄)₂SO₄ in the buffer. Indeed, the accessibility of Cys-382 is also strongly increased, whereas under similar conditions of salts, the accessibility of Cys-382 in apo-wild type remains low (results not shown). In the presence of saturating concentration of NADP, under conditions where the conformational transition upon cofactor binding has occurred (see below), the pH dependence curve exhibited a monosigmoidal profile with a pK_{app} of 6.7 and a low k_{app} value of 97 M⁻¹s⁻¹ (Fig. 3A, open symbols). The fact that the observed pK_{app} of Cys-302 is 2 pH units lower than that of Cys-382 explains why only Cys-302 was titrated under the pH range tested.

As indicated above, K_m value for D-G3P of R301L mutant is higher than 15 mM, and the k_{app}/K_m value is drastically decreased. As a consequence, at the concentrations of D-G3P used, the absorption signal at 340 nm is low, particularly at pH lower than 7. Therefore, it was not possible to trace a pH-k_{app}/K_m curve profile with sufficient confidence to attain pK_{a} values. On the other hand, the kinetics with 2PDS could be attained. Kinetics were carried out under similar conditions to those described with the R459I mutant GAPN protein except that 0.25 M of (NH₄)₂SO₄ was omitted in the reaction buffer. Two moles of pyridine-2-thione per subunit were formed with both apo form and apo form on which NADP was bound. The pH-k_{2} curves obtained for both forms fitted better to a monosigmoidal profile with pK_{app} values of 8.5 and k_{app} values of 450 M⁻¹s⁻¹ and 91 M⁻¹s⁻¹, respectively (see Fig. 3B, solid and dotted lines). These data indicate that 1) both Cys-302 and Cys-382 are little accessible and have similar pK_{app} values and 2) NADP binding to R301L GAPN is not sufficient to induce a conformational rearrangement of the active site in contrast to that observed for the wild type (14).

Characterization of the Charge Transfer Transition in Holo-R459I Mutant GAPN—As shown in Fig. 4A, the binding of NADP to apo-R459I mutant led to formation of an absorption band at 333 nm, called Ab₃₃₃. This is a situation similar to that observed with phosphorylating NAD-dependent glyceraldehyde-3-phosphate dehydrogenase. In this latter enzyme, it was shown that the transition was due to a charge transfer between the essential Cys-149 and the nicotinamide ring of the cofactor acting as an electron donor and electron acceptor, respectively (20, 21). Therefore, it was reasonable to hypothesize that Ab₃₃₃ was due to a charge transfer between Cys-302 and the nicotinamide of NADP. The fact that Ab₃₃₃ disappeared in the presence of 10 mM iodoacetamide (Fig. 4A), which is a probe of cysteine residue, strengthens this assumption. At pH 8.5, Ab₃₃₃ showed a maximal absorbance with a molar extinction coefficient ε₃₃₃ of 6500 M⁻¹cm⁻¹. A study of the pH dependence of the intensity of Ab₃₃₃ absorbance also revealed an inflection point at pH 6.6 (Fig. 4A). Therefore, this pH corresponds to the pK_{app}...
of Cys-302. This also correlates well with the $pK_{app}$ value found by titration with 2PDS. Moreover, the appearance of Ab333 was determined using nonlinear regression analysis, and second order rate constants $k_2$ were calculated by dividing values of $k_{obs}$ by the concentration of 2PDS. For both mutants, $k_2$ fitted to Equation 3. A. $pK_{app}$ values of 8.5 and higher than 9.2 were determined for the R459I mutant apoenzyme (○, ■, respectively), and a $pK_{app}$ of 6.7 for the holoenzyme (○). B. $pK_{app}$ values of 8.5 were deduced from the best fit for the R301L mutant apoenzyme (○, ■, solid lines) and NADP-bound apoenzyme (○, □, dotted lines).

Fig. 3. pH dependence of the second-order rate constant ($k_2$) for the reaction of 2PDS on R459I (A) and R301L (B) mutants. Kinetics were performed at 25 °C over a pH range of 5–9.5, as indicated under “Experimental Procedures.” The concentrations of mutants and 2PDS were 3.71 μM (in sites) and 111 μM, respectively. Rate constants, $k_{obs}$, were determined using nonlinear regression analysis, and second order rate constants $k_2$ were calculated by dividing values of $k_{obs}$ by the concentration of 2PDS. For both mutants, $k_2$ fitted to Equation 3. A. $pK_{app}$ values of 8.5 and higher than 9.2 were determined for the R459I mutant apoenzyme (○, ■, respectively), and a $pK_{app}$ of 6.7 for the holoenzyme (○). B. $pK_{app}$ values of 8.5 were deduced from the best fit for the R301L mutant apoenzyme (○, ■, solid lines) and NADP-bound apoenzyme (○, □, dotted lines).

FIG. 4. Properties of the charge transfer transition in holo-R459I mutant. A. pH dependence of Abs333 absorbance. Inset, effect of the addition of iodoacetamide (in excess of 50-fold relative to the subunit enzyme concentration) on the Ab333 absorbance. B, pH dependence of the rate of the Ab333 appearance. The dashed lines represent $h$ values of each protonic state provided by the individual terms of Equation 6. The first $pK_{app}$ value of 6.6 corresponds to that of Cys-302, and the second one of 7.6 corresponds to that of Glu-268. See “Experimental Procedures” and “Results” for more details.

apo structure facilitates the conformational change at least within the binary complex GAPN-NADP.

DISCUSSION

GAPN belongs to the ALDH family. It is one of the ALDHs whose properties have been the most studied at both the structural and enzymatic levels. The fact that all of these studies were carried out with the physiological substrate d-G3P is also a guarantee of the generality of the interpretations of the results. Taking into account all of the enzymatic and structural data available so far, a scenario of the catalytic mechanism of GAPN from S. mutans can be described as presented in the Introduction. As shown, the binding of NADP to apo-GAPN induces a local conformational change of the active site with at least a reorientation of side chains of Cys-302, Glu-268, and Arg-459. The rate of the conformational change upon cofactor binding has a maximal $k_{obs}$ value of $4.7 \times 10^{-4}$ s$^{-1}$ at acidic pH, which is not compatible with the $k_{ac}$ and $k_{cat}$ values (14). The fact that adding d-G3P increases the rate of the reorganization by a factor of at least 10$^3$-fold (14) indicates that d-G3P binding to the binary complex GAPN-NADP strongly favors the active site reorganization. Therefore, characterizing the structural factors involved in d-G3P binding could help to a better understanding of the catalysis and of the evolution of the substrate binding sites of ALDHs. As presented in the Introduction, four residues seem to be conserved in the GAPN family (i.e. Arg-124, Tyr-170, Arg-301, and Arg-459) and could be involved in the recognition of d-G3P and/or in catalysis.

Substituting Arg-124 with leucine does not change the nature of 1) the rate-limiting step, which remains deacetylation, and 2) the rate-determining step in acylation, which remains

$^6$S. Marchal and G. Branlant, unpublished results.
hydride transfer. Moreover, no significant effect is observed on acylation and deacylation rates. Therefore, these results exclude a role of the guanidinium group of Arg-124 in stabilizing the transition states associated with hydride transfer and involved in hydrolysis. Only an increase in $K_m$ is observed in both acylation and deacylation steps but with a more pronounced effect in the latter step. Therefore, the only role of Arg-124 is to stabilize the binding of G3P via an interaction between the guanidinium group and the phosphate at C-3. This is in accord with the structure of 1) the ternary complex C302S-NADP-D-G3P in which the guanidinium group of Arg-124 interacts with one of the oxygens of the phosphate and 2) the apo2 form in which the guanidinium group interacts with one of the oxygens of the anion $SO_4a$ that is postulated to mimic the phosphate of n-G3P. The fact that the $pK_{app}$ of Cys-302 within the acylation complex remains similar to that observed in wild type indicates no role of Arg-124 in activating Cys-302. Again, this is in accord with the x-ray structure of apo2 GAPN, which shows a distance between both residues higher than 12 Å.

The situation is different for the Y170F mutant GAPN protein. No major effect is observed in the $K_m$ values for n-G3P and in the rates of both acylation and deacylation steps. Therefore, although the hydroxy group of Tyr-170 within the ternary complex C302S-NADP-D-G3P has been shown to be at a hydrogen bonding distance of the oxygen that connects the C-3 carbon to the phosphate in n-G3P, the interaction seems not to be essential for n-G3P binding. The fact that, in some archeal GAPNs, Tyr-170 is replaced by a phenylalanine supports the present results (Fig. 1).

The behavior of the R459I mutant GAPN protein is very different. With this, the acylation process becomes rate-limiting with a limiting step prior to hydride transfer. The efficiency of the acylation step is strongly decreased by at least a factor of $10^5$, which includes both a $K_m$ and a $k_{on}$ contributions. From different approaches, i.e. using kinetic probes like 2PDS and the $Ab_{333}$ or tracing the pH rate profile of acylation and of Arg-459, which is situated 5.7 Å within either the holo form or the ternary complex. Therefore, indirectly in lowering the $pK_{app}$, Arg-459 main chain NH interacts with the $SO_4a$, which is representative of the phosphate of n-G3P, whereas guanidinium side chain interacts with $SO_4b$, which probably mimics the $sp_3$ transition states. Arg-459 interacts also with Gln-455, which is a residue conserved in almost all GAPNs. Therefore, substituting Arg-459 by isoleucine can also modify the conformation of the loop on which Arg-459 is located. Together, this is in accord with the data of the present study, which shows that substituting Arg-459 by isoleucine has not only a destabilizing $K_m$ effect for n-G3P but also a strong kinetic effect, which reflects a Michaelis ternary complex that seems to be inefficient for forming the thiohemiacetal intermediate ternary complex.

The role of Arg-301 is more difficult to comprehend in detail due to the very low catalytic efficiency of R301L mutant GAPN protein. As with the R459I mutant, the rate-limiting step is associated with acylation, more precisely prior to hydride transfer, and a high $K_m$ value for n-G3P and a low $k_{cat}$ value are observed. Taking into account the apo2 structure of GAPN, which shows an interaction of Arg-301 guanidinium side chain with both the $SO_4a$ and $SO_4b$, it is probable that substituting Arg-301 by leucine should destabilize n-G3P binding but at the same time perturb the relative positioning of n-G3P and of Cys-302 within the ternary complex. Consequently, this would prevent an efficient attack of the thiolate group toward the aldehydic group.

GAPN from S. mutants reduces efficiently D and L isomers of G3P (16). Therefore, Arg-301 and Arg-459 are probably not involved in G3P stereospecificity. This suggests that both arginines do not interact selectively with the C-2 moiety of n-G3P. This is also supported by the observation that both residues are conserved in GAPNs from plants which are described to reduce only D isomer (22). Thus, as supported by the present study, one major role of Arg-301 and Arg-459 would be to position efficiently the D-G3P with respect to Cys-302 within the Michaelis ternary complex. This would favor the formation of the thiohemiacetal intermediate and consequently the efficiency of the hydride transfer. During this part of the catalytic event, the geometry at the C-1 carbon changes from $sp_2$ to $sp_3$ and reveals the role of Asn-169. Now, the negative charge of the oxygen of the thiohemiacetal intermediate is stabilized by the oxygen site, which is composed at least of the amide side chain of Asn-169. This is in accord with previous studies, which showed that mutating Asn-169 results in a drastic decrease of the hydride transfer rate but has no effect on $K_m$ value for n-G3P (15).

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References
1. Lindahl, R. (1992) Crit. Rev. Biochem. Mol. Biol. 27, 283–335
2. Vasilieou, V., Pappa, A., and Petersen, D. R. (2000) Chem. Biol. Interact. 129, 1–19
3. Vasilieou, V., and Pappa, A. (2000) Pharmacology 61, 192–198
4. Klyosov, A. A., Rashkovetsky, L. G., Tabir, M. K., and Keang W. M. (1996) Biochemistry 35, 4445–4456
5. Klyosov, A. A. (1996) Biochemistry 35, 4457–4467
6. Li, Z.-J., Sun, Y.-J., Rose, J., Chang, Y. J., Haiz, C.-D., Chang, W.-R., Kao, L., Pehozich, J., Lindahl R, Hempel, J., and Wang B. C. (1997) Nat. Struct. Biol. 4, 317–326
7. Steinmetz, C. G., Xie, P., Weiner, H., and Hurley, T. D. (1997) Structure 5, 701–711
8. Moore, S. A., Baker, H. M., Blythe, T. J., Kitson, K. E., Kitson, T. M., and Baker, E. N. (1998) Structure 6, 1541–1551
9. Lamb, A. L., and Newcomer, M. E. (1999) Biochemistry 38, 6003–6011
10. Johansson, K., El-Ahmad, M., Ramaswamy, S., Hjelmqvist, L., Jornvall, H., and Eklund, H. (1998) Protein Sci. 7, 2106–2117
11. Li, Z., Zhou, J., Hurley, T. D., and Weiner, H. (1999) Protein Sci. 8, 2784–2790
12. Alhaz, B., Coulombe, R., Delarge, M., Vredin, D., Zhang, L., Meigten, E., and Vredin, A. (2000) Biochem. J. 349, 853–861
13. Woit, E., Brunner, N., Wilmanns, M., and Henschel, R. (2002) J. Biol. Chem. 277, 19938–19945
14. Marcell, S., and Brantlan, G. (1999) Biochemistry 38, 12950–12958
15. Cobuei, D., Teie-Favere, F., Marcell, S., Brantlan, G., and Aubry, A. (2000) J. Mol. Biol. 306, 141–152
16. Marcell, S., Rahuel-Clermont, S., and Brantlan, G. (2000) Biochemistry 39, 3327–3335
17. Ni, L., Sheikh, S., and Weiner, H. (1997) *J. Biol. Chem.* **272**, 18823–188236
18. Cobessi, D., Tête-Favier, F., Marchal, S., Azza, S., Branlant, G., and Aubry, A. (1999) *J. Mol. Biol.* **290**, 161–173
19. Corbier, C., Mougin, A., Mely, Y., Adolph, H. W., Zeppezauer, M., Gerard, D., Wonacott, A., and Branlant, G. (1990) *Biochimie (Paris)* **72**, 545–554
20. Racker, E., and Krimsky, L. (1952) *J. Biol. Chem.* **198**, 731–743
21. Mougin, A., Corbier, C., Soukri, A., Wonacott, A., Branlant, C., and Branlant, G. (1988) *Protein Eng.* **2**, 45–48
22. Gomez Casati, D. F., Sesma, J. I., and Iglesias, A. A. (2000) *Plant Sci.* **154**, 107–115
23. Wang, X., and Weiner, H. (1995) *Biochemistry* **34**, 237–243
24. Hochachka, P. W., and Somero, G. N. (2002) *Biochemical Adaptation: Mechanism and Process in Physiological Evolution*, pp. 290–316, Oxford University Press, New York