Comparative Enzymatic Properties of GapB-encoded Erythrose-4-Phosphate Dehydrogenase of *Escherichia coli* and Phosphorylating Glyceraldehyde-3-phosphate Dehydrogenase*

(Received for publication, January 27, 1997, and in revised form, March 19, 1997)

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GapB-encoded protein of *Escherichia coli* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) share more than 40% amino acid identity. Most of the amino acids involved in the binding of cofactor and substrates to GAPDH are conserved in GapB-encoded protein. This enzyme shows an efficient non-phosphorylating erythrose-4-phosphate dehydrogenase activity (Zhao, G., Pease, A. J., Bharani, N., and Winkler, M. E. (1995) *J. Bacteriol.* 177, 2804–2812) but a low phosphorylating glyceraldehyde-3-phosphate dehydrogenase activity, whereas GAPDH shows a high efficient phosphorylating glyceraldehyde-3-phosphate dehydrogenase activity and a low phosphorylating erythrose-4-phosphate dehydrogenase activity. To identify the structural factors responsible for these differences, comparative kinetic and binding studies have been carried out on both GapB-encoded protein of *Escherichia coli* and GAPDH of *Bacillus stearothermophilus*. The $K_D$ constant of GapB-encoded protein for NAD is 800-fold higher than that of GAPDH. The chemical mechanism of erythrose 4-phosphate oxidation by GapB-encoded protein is shown to proceed through a two-step mechanism involving covalent intermediates with Cys-149, with rates associated to the acylation and deacylation processes of 280 s$^{-1}$ and 20 s$^{-1}$, respectively. No isotopic solvent effect is observed suggesting that the rate-limiting step is not hydrolysis. The rate of oxidation of glyceraldehyde 3-phosphate is 0.12 s$^{-1}$ and is hydride transfer limiting, at least 2000-fold less efficient compared with that of erythrose 4-phosphate. Thus, it can be concluded that it is only the structure of the substrates that prevails in forming a ternary complex enzyme-NAD-thiohemiacetal productive (or not) for hydride transfer in the acylation step. This conclusion is reinforced by the fact that the rate of oxidation for erythrose 4-phosphate by GAPDH is 0.1 s$^{-1}$ and is limited by the acylation step, whereas glyceraldehyde 3-phosphate acylation is efficient and is not rate-determining (800 s$^{-1}$). Substituting Asn for His-176 on GapB-encoded protein, a residue postulated to facilitate hydride transfer as a base catalyst, decreases 40-fold the $k_{cat}$ of glyceraldehyde 3-phosphate oxidation. This suggests that the non-efficient positioning of the C-1 atom of glyceraldehyde 3-phosphate relative to the pyridinium of the cofactor within the ternary complex is responsible for the low catalytic efficiency. No phosphorylating activity on erythrose 4-phosphate with GapB-encoded protein is observed although the Pi site is operative as proven by the oxidative phosphorylation of glyceraldehyde 3-phosphate. Thus the binding of inorganic phosphate to the Pi site likely is not productive for attacking efficiently the thioacyl intermediate formed with erythrose 4-phosphate, whereas a water molecule is an efficient nucleophile for the hydrolysis of the thioacyl intermediate. Compared with glyceraldehyde-3-phosphate dehydrogenase activity, this corresponds to an activation of the deacylation step by 4.5 kcal mol$^{-1}$. Altogether these results suggest subtle structural differences between the active sites of GAPDH and GapB-encoded protein that could be revealed and/or modulated by the structure of the substrate bound. This also indicates that a protein engineering approach could be used to convert a phosphorylating aldehyde dehydrogenase into an efficient non-phosphorylating one and vice versa.

The glycolytic glyceraldehyde-3-phosphate dehydrogenase (GAPDH)$^1$ is a tetrameric enzyme that catalyzes reversibly the oxidative phosphorylation of D-glyceraldehyde 3-phosphate (G3P) to form 1,3-diphosphoglycerate (1,3-dPG) in the presence of NAD and inorganic phosphate (1). The refined structures of several phosphorylating GAPDHs have already been reported (2–7). The currently accepted forward reaction pathway involves two steps, first the formation of a covalent ternary complex GAPDH-NAD-G3P preceding an oxidoreduction step that leads to a thioacyl enzyme intermediate and NADH, and second the phosphorylation that produces 1,3-dPG. The chemical mechanism of catalysis was extensively studied and is now well understood (8, 9). The structural determinants of the nicotinamide and adenosine subsites involved in coenzyme specificity that control the catalytic efficiency have been recently analyzed (10–13). The individual contribution of the amino acids implicated in the two phosphate binding sites named Ps and Pi sites has also been studied at the kinetic, structural, and energetic levels (14, 15).

GAPDH is a key enzyme of the glycolysis and gluconeogenesis pathways. In the gram *Escherichia coli*, three distinct gap genes have been isolated so far. The gapA gene was shown to encode an efficient active GAPDH (16, 17). A second gap gene, $^{1}$The abbreviations used are: GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; G3P, glyceraldehyde 3-phosphate; 1,3-dPG, 1,3-diphosphoglycerate; E4P, erythrose 4-phosphate; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

$^*$ This research was supported by the Center National de la Recherche Scientifique and the University Henri Poincaré Nancy I. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a fellowship from the Association de Recherche Contre le Cancer.

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called gapB, was initially characterized by Alefounder and Perham (18). This gene belongs to a cluster of genes coding for several glycolytic enzymes. This gene organization is often found in eubacteria and archaeabacteria studied so far (Ref. 17 and references cited therein) and is usually responsible for the GAPDH activity. However, a peculiar situation is observed in *E. coli*. The gapB gene, which is located within the glycolytic gene cluster, does not seem to encode GAPDH activity (18). Indeed, Hillman and Fraenkel (19) showed that a nonsense mutation in the gapA gene abolishes GAPDH activity. Thus, the GAPDH activity is only encoded by the gapA gene that is 22.6 min far from the phosphoglycerate kinase gene on the *E. coli* chromosome. This has led to the proposal of either an eukaryotic origin for the gapA gene (18, 20, 21) or the presence of both gapA and gapB genes in ancestor bacteria (22). A third gene named gapC was recently characterized.\(^2\) The presence of several stop codons in the putative coding regions, clearly indicates that gapC gene can not encode a functional GAPDH.

Amino acid sequence comparison of GAPDHs from *E. coli* and *Bacillus stearothermophilus* and GapB\(^3\)-encoded protein shows 41.6 and 43.6% identity, respectively. Nearly all the amino acids essential for the chemical mechanism and for the binding of cofactor and substrates are conserved. In the present paper, the enzymatic properties of the protein encoded by the gapB gene are described and compared with those of the GAPDH from *B. stearothermophilus*. The results are discussed in relation to the three-dimensional structures of the *E. coli* (7) and *B. stearothermophilus* (3) GAPDHs and to the recent data of Zhao et al. (23), who showed that GapB-encoded protein displays an efficient non-phosphorylating erythrose-4-phosphate dehydrogenase activity.

**MATERIALS AND METHODS**

*Site-directed Mutagenesis, Production, and Purification of Wild-type and Mutant Enzymes*—The *E. coli* strain used for wild-type and mutant GapB-encoded protein production was HB101 (F\(^-\), supE44, hsdR17, recA13, ara-14, proA2 lacY1, galK2, rpsL20, xyl-5, met-l, strA1) transformed with a plasmidic construction containing the gapB gene under either its own promoter or the gapA promoter.\(^4\) Site-directed mutagenesis was performed using the method of Kunkel et al. (24). Cells were harvested by centrifugation after denaturation by addition of H\(_2\)SO\(_4\). The solution was adjusted to pH 10.5 by addition of NaOH and sonicated. The GapB-encoded protein was then isolated by a 45% (NH\(_4\))\(_2\)SO\(_4\) precipitation. The contaminating proteins were removed by applying the enzymatic solution onto gel filtration, ACA 34 resin at pH 8 (buffer A). The enzyme was then purified on a Q-Sepharose column equilibrated with buffer A, followed by a linear gradient of KCl (0–0.5 M) for GapB-encoded protein and 0–5 mM for GAPDH. The concentration of ion exchange was calculated using an extinction coefficient at 280 nm of 1.50 × 10\(^4\) M\(^-1\) cm\(^-1\) for the GapB-encoded protein and of 1.31 × 10\(^4\) M\(^-1\) cm\(^-1\) for the GAPDH of *B. stearothermophilus*. K\(_{\text{m}}\) is expressed per site.

The initial rate data were fitted to the Michaelis-Menten relationship using least squares regression analysis to determine V\(_{\text{m}}\) and K\(_{\text{m}}\). All Km values were determined at saturating concentrations of the other substrates.

**Purification of E4P**—Commercially available erythrose 4-phosphate (E4P) (Sigma) contained low levels of G3P, glucose 6-phosphate, and other contaminants (23). The true concentration of E4P was deduced from the amount of NADH formed under experimental conditions in which the E4P dehydrogenase reaction proceeded to completion. The average purity was estimated to be 60% as described by the manufacturer.

To isolate pure E4P, contaminant G3P was oxidized by GAPDH from *B. stearothermophilus* in the presence of phosphate and NAD, and a coupled system using lactate dehydrogenase was used for NADH recycling to displace the equilibrium of the reaction in favor of the formation of 1,3-dPG. The time of the reaction was chosen to oxidize totally G3P without significant oxidation of E4P. Enzymes were then removed by centrifugation after denaturation by addition of H\(_2\)SO\(_4\). The solution was then diluted in imidazole buffer, pH 7, and applied onto a DEAE column (LKB5 PW) previously equilibrated in 25 mM imidazole buffer, pH 7. Elution was performed with a linear gradient of KCl (0–0.5 M) that allowed easy separation of E4P from 1,3-dPG. The content in E4P of the fractions was measured enzymatically using GapB-encoded protein and NAD in 40 mM triethylamine buffer, 2 mM EDTA, pH 8.9.

**Titration of the Cysteine Residues**—The cysteine content was determined using DTNB under non-denaturating and denaturing conditions. Progress curves of thionitrobenzoate production for wild-type and mutant enzymes were recorded at 412 nm in 50 mM Tris-HCl buffer, 2 mM EDTA, pH 8.2, for non-denaturing conditions. For denaturing conditions, SDS was added (10% final) and enzyme was heated 10 min at 70 °C. Enzyme concentration was 14.7 μM (in nondenaturing conditions) of 0.5 μM final. The amount of thionitrobenzoate formed was calculated using an extinction coefficient at 412 nm of 13,600 M\(^-1\) cm\(^-1\).

**Determination of Apparent Dissociation Constant for NAD and NADH**—Iodoacetamide was used as a second order labeling probe of cysteine 149 by measuring the protection against inactivation afforded by the coenzyme. Inactivation of GapB-encoded protein (5 μM) and GAPDH (0.2 μM) was assayed in 0.1 M TES buffer, EDTA 0.2 mM, pH 7.3. The concentration of iodoacetamide was 100 μM. The concentration of NAD was varied over the range 0–7.5 mM for GapB-encoded protein and 0–5 μM for GAPDH. The concentration of NADH was varied over the range 0–750 μM for GapB-encoded protein and 0–5 μM for GAPDH. Aliquots were withdrawn from the incubation mixture at fixed intervals, and the residual activity was then determined by dilution into the assay mixture. Assumption was made that the chemical reactivity of cysteine 149 is similar in the apo-form and the holo-form. The pseudo-first-order constant k\(_{\text{obs}}\) was determined for each NAD or NADH concentration from plots of log(ΔA/Δt) versus time (A\(_0\) and A\(_t\) correspond to the initial enzymatic activity and the activity at time t, respectively). Dissociation constant (K\(_D\)) of NAD or NADH was determined from plots of (1 – k\(_{\text{obs}}\)/k\(_{\text{app}}\)) (NAD) versus k\(_{\text{obs}}\)/k\(_{\text{app}}\) and k\(_{\text{obs}}\)/k\(_{\text{app}}\) correspond to the pseudo-first-order constant k\(_{\text{obs}}\) in the absence and presence of NAD or NADH, respectively.

**Stopped-flow Kinetic Measurements**—Fast kinetic measurements were carried out on a Biologic Instruments (SPFM3) stopped-flow apparatus. The dead time of the apparatus under the conditions of flow rate and measurement cell employed was determined using reduction of 2,6-dichlorophenol by ascorbic acid (28). It was estimated at 1.4 ms while the apparent first order rate constant of the reaction was proportional to the ascorbic acid concentration until about 900 s\(^-1\), thus setting the upper limit of reliable measurements. Data collected from absorbance measurements at 25 °C were analyzed with the Biokin program using non-linear regression analysis. An average of at least six runs was performed to determine each constant.

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\(^2\) E. Hidalgo, A. Limon, and J. Aguilar, unpublished results.

\(^3\) To avoid misunderstanding, GAPDH refers to protein having high activity with G3P. The protein encoded by the gapB gene, which showed low GAPDH activity, is named GapB-encoded protein.

\(^4\) B. Charpentier, unpublished results.
Analysis of the steps leading to thioacyl enzyme formation was performed as described previously (14) except that G3P was replaced by E4P at final concentration of 2 mM.

Analysis of the alkylation of Cys-149 by 5,5'-dithiobis-(2-nitrobenzoate) was made under nondenaturing conditions. Progress curves of thionitrobenzoate production for wild-type and mutant enzymes were recorded at 412 nm in 50 mM Tris-HCl buffer containing 2 mM EDTA, pH 8.2. One syringe was filled with enzyme (14.7 μM), and the other one contained DTNB (600 μM).

Enzymatic and Chemical Characterization of the Reaction Products—Oxidation of G3P by GapB-encoded protein was done in the absence and presence of phosphate (100 mM). Oxidized products were isolated and enzymatically characterized as already described (9). The chemical nature of the reaction products was also confirmed by 31P NMR. Purified products were lyophilized and dissolved in D2O, and the pH was adjusted to 7. NMR spectral data were obtained on a Bruker AC250 spectrometer. Chemical shifts were referenced to external phosphoric acid at 85%. The signals of the C-3 phosphate were 4.47, 3.37, and 3.94 ppm for G3P, 3-phosphoglycerate, and 1,3-dPG respectively, and that of the C-1 phosphate of 1,3-dPG was 2.21 ppm. The chemical shift of free inorganic phosphate was 2.60 ppm.

Oxidation of E4P by GapB-encoded protein and GAPDH was also done in the absence and presence of phosphate (100 mM). Oxidized products were isolated and enzymatically characterized as already described (9). The chemical nature of the reaction products was also confirmed by 31P NMR spectroscopy (chemical shift of 4-phosphoerythronate, 4.13 ppm).

Deuterium Isotope Effects—D-G3P and D-[1-2H]G3P were prepared as described previously (11). Their concentrations were enzymatically determined. Isotopic effects were measured in 40 mM triethanolamine buffer, 2 mM EDTA, pH 8.9, by direct comparison of initial velocities observed for oxidative phosphorylation of D-G3P and D-[1-2H]G3P (1 mM) in the presence of 10 mM NAD and 50 mM phosphate.

RESULTS

Justification of the Mutations

Site-directed mutagenesis at positions 32, 149, 176, 179, 206–209, and 311 were done for the following reasons (see also Fig. 1).

Fig. 1. Amino acid sequence alignment of GapB-encoded protein and GAPDHs. The GapB-encoded protein sequence is from Alefounder and Perham (18). The numbering of amino acid residues is according to Biesecker et al. (31). The strictly conserved amino acids in all GAPDHs (>95%) are illustrated in boldface. A total of 95 GAPDHs with known primary structures from the Swiss-Prot and Pir data bases have been taken into account. Sequence alignment was performed with the program Alscript (32). The secondary structures (α and β for helix and β-sheet, respectively) are indicated on the basis of the crystalline structures of B. stearothermophilus (3) and E. coli (7) GAPDHs.
Position 311—This position is always occupied by a Tyr residue in all GAPDHs described so far except for the GapB-encoded protein where a Cys residue is present. Tyr-311 is at the hinge between catalytic and cofactor domains and is located near the catalytic amino acids Cys-149 and His-176 and also near Cys-153 (see Fig. 7 in Ref. 7). Modeling cannot exclude the possibility for Cys-311 to form an alternative thioacyl enzyme intermediate with E4P that has an additional CHO group compared with G3P.

Position 179—This position belongs to the Ps site and is always occupied by a Thr in all active GAPDHs described so far (15) except for the GapB-encoded protein where a Met residue is present (18).

Position 32—This position is always occupied by an Asp residue, which forms a hydrogen bond with both 2'- and 3'-hydroxyl groups of the ribose of the adenosine moiety, except for the GapB-encoded protein where a Glu residue is present. Since the substitution Asp-32 → Glu in B. stearothermophilus GAPDH increased 9-fold the $K_M$ of NAD (12), the presence of a Glu-32 could be one of the factors responsible for the low affinity of NAD(H) of the GapB-encoded protein (see “Results”).

Position 206–209—This sequence contained positions always invariant in GAPDH that are involved in the Pi site, i.e. Thr-208 and Gly-209. This sequence is largely changed in the GapB-encoded protein, where in particular Gly-209 is substituted by a Lys residue (see Fig. 1).

Biochemical Properties of Wild-type and Mutated GapB-encoded Protein

GapB-encoded protein was overexpressed in E. coli strain using a plasmidic construction containing the gapB gene under either its own promoter or the gapA promoter. Over 15% of the soluble proteins in the supernatant were GapB-encoded proteins. The protocol used to purify GapB-encoded protein to homogeneity took advantage of the higher hydrophobic character of GapB-encoded protein compared with the GAPDH from E. coli. This allowed easy separation of GapB-encoded protein from GTA encoded protein, where in particular Gly-209 is substituted by a Lys residue (see Fig. 1).

| Fraction                  | Total protein | Specific activity (μmol/min mg) | Total activity (μmol/min) | Purification factor | Yield (%) |
|---------------------------|---------------|-------------------------------|--------------------------|---------------------|-----------|
| Homogenate                | 8957          | 0.14                          | 1279                     | 1                   | 100       |
| (NH$_4$)$_2$SO$_4$ 3947    | 3847          | 0.27                          | 1066                     | 2                   | 83        |
| ACA 34                    | 363           | 2.54                          | 849                      | 17                  | 66        |
| Q-Sepharose               | 52            | 14.30                         | 742                      | 102                 | 58        |
| Phenyl-Sepharose          | 50            | 14.70                         | 735                      | 105                 | 57        |

With G3P as a Substrate—The kinetic parameters ($k_{cat}$ and $K_M$) of GapB-encoded protein and its various mutants are summarized in Table II. A 600-fold decrease in the $k_{cat}$ value in the forward reaction was observed for the GapB-encoded protein compared with the GAPDH from B. stearothermophilus. $K_M$ value for NAD increased 10-fold. To determine the nature of the limiting step and to define whether the catalytic mechanism proceeded via two distinct chemical steps as shown for GAPDH, transient kinetics in the absence or presence of inorganic phosphate (50 mM) were carried out at saturating NAD concentrations. Under these experimental conditions no burst of NADH production was observed. The rate constant of 0.12 s$^{-1}$ is similar to the $k_{cat}$ value obtained under steady state conditions regardless of the presence or absence of inorganic phosphate (curves not shown). This demonstrated that the limiting step is associated with the formation of the acyl enzyme intermediate and not with steps occurring after the acylation, as shown for the GAPDHs from E. coli and B. stearothermophilus (11, 14). The foregoing results indicated an acylation step at least 7000-fold less efficient compared with the GAPDH from B. stearothermophilus. The presence of an isotopic effect of 5 with D-[3-2H]G3P as a substrate (data not shown) demonstrated that the rate-limiting step is associated with the hydride transfer.

The fact that the acylation was limiting did not exclude an efficient phosphorylating step. To investigate this possibility, the chemical nature of the product formed in the forward direction in the absence and presence of 0.1 M phosphate was determined enzymatically and characterized chemically by $^{31}$P NMR. It was shown to be 3-phosphoglycerate and 1,3-dPG, respectively.
Erythrose 4-Phosphate Dehydrogenase Activity

Table II

Kinetic parameters of wild-type and mutants of GapB-encoded protein, and wild-type and mutant of B. stearothermophilus GAPDH

| Substrate | G3P | E4P |
|-----------|-----|-----|
|           | \(K_M\) G3P | \(K_M\) NAD | \(k_{cat}\) | \(K_M\) E4P | \(K_M\) NAD | \(k_{cat}\) |
|           | \(\text{mM}\) | \(\text{mM}\) | \(s^{-1}\) | \(\text{mM}\) | \(\text{mM}\) | \(s^{-1}\) |
| Wild-type GapB-encoded protein | 1.10 ± 0.50 | 0.90 ± 0.20 | 0.12 ± 0.04 | 0.51 ± 0.09 | 0.8 ± 0.2 | 20 ± 1 |
| E22D mutant | 1.50 ± 0.20 | 1.10 ± 0.20 | 0.10 ± 0.05 | 1.90 ± 0.20 | 2.3 ± 0.4 | 7 ± 0.4 |
| H179N mutant | 3.00 ± 1.00 | 0.72 ± 0.04 | 0.0030 ± 0.0006 | 0.24 ± 0.04 | 0.5 ± 0.1 | 0.21 ± 0.01 |
| M179T mutant | 1.00 ± 0.40 | 1.00 ± 0.20 | 0.11 ± 0.03 | 0.70 ± 0.10 | 3.5 ± 0.6 | 4.0 ± 0.4 |
| C311A mutant | 0.41 ± 0.08 | 1.00 ± 0.10 | 0.035 ± 0.003 | 0.80 ± 0.10 | 4 ± 1 | 3.5 ± 0.5 |
| C311Y mutant | 0.30 ± 0.10 | 2.20 ± 0.70 | 0.020 ± 0.003 | 0.70 ± 0.30 | 5 ± 2 | 0.6 ± 0.1 |
| Wild-type B. st. GAPDH | 0.90 ± 0.20 | 0.09 ± 0.01 | 76 ± 4 | 3.30 ± 1.10 | 0.08 ± 0.02 | 0.10 ± 0.02 |
| GAPDH T197M mutant* | 0.16 ± 0.03 | 0.31 ± 0.05 | 0.015 ± 0.005 | ND | ND | ND |

* From Michaels et al. (15).

(see “Materials and Methods”). Thus, the GapB-encoded protein exhibits a phosphorylating activity more efficient than the non-phosphorylating one. Clearly, the Pi site is operational. In the reverse direction, 1,3-dPG was shown to be a substrate with a \(K_M\) value increased 16-fold, a \(K_M\) value of NADH increased 5-fold, and a \(k_{cat}\) decreased 880-fold compared with GAPDH (\(K_M\) (1,3-dPG) 0.08 and 0.005 mM, \(K_M\) (NADH) 0.05 and 0.011 mM and \(k_{cat}\) 0.074 s\(^{-1}\) and 65 s\(^{-1}\) for GapB-encoded protein and GAPDH of B. stearothermophilus, respectively).

As shown in Table II, replacing Met-179 by Thr and Cys-311 by Tyr or Ala did not drastically change the GAPDH activity of GapB-encoded protein, whereas changing His-176 into Asn decreased \(k_{cat}\) by a factor of 40. No significant activity was observed for C149G, A or V mutants under the experimental conditions used (data not shown, see “Discussion”).

With E4P as a Substrate—The data confirmed those already published by Zhao et al. (23) showing an E4P dehydrogenase activity. A \(k_{cat}\) of 20 s\(^{-1}\) and a \(K_M\) of 800 \(\mu\)M for NAD were found under optimal conditions. The \(k_{cat}\) value is 2.5-fold smaller than that described previously (23). This discrepancy remains to be explained. An E4P dehydrogenase activity for C149G, A and V mutants, purified from a E. coli strain, was observed at least 2000-fold less compared with wild type. This low activity is in fact due to the GapB-encoded protein expressed from the chromosomal gapB gene of E. coli strain used to overexpress the mutants. Indeed the mutants purified from a gapB gene by a factor of 1000-fold less compared with wild type (data not shown). The fact that CD spectra of the mutants and wild type perfectly superposed (curves not shown) suggests that the absence of activity of the mutants is not a consequence of change in their structure.

Replacing amino acids located in the catalytic subsite, i.e. Met-179 by Thr, Cys-311 by Ala or Tyr, His-176 by Asn reduced \(k_{cat}\) to that used for 1,3-dPG (9) and characterized enzymatically by measuring the protection against inactivation afforded by iodoacetamide as a second order labeling probe of cysteine by measuring the protection against inactivation afforded by the coenzyme binding. The deduced \(K_D\) value provides a measure of macroscopic binding of NAD. Surprisingly, the NAD and NADH dissociation constants \(K_D\) increased 800- and 300-fold, respectively, compared with GAPDH (see Table III).

This can also be related to the observation that GapB-encoded protein is always isolated as an apo form. The low NAD affinity is in fact due to the GapB-encoded protein expressed from the strain used to overexpress the mutants.

To determine whether the higher \(K_M\) value for NAD is indicative of a decrease of its affinity, \(K_D\) value was determined using iodoacetamide as a second order labeling probe of cysteine by measuring the protection against inactivation afforded by the coenzyme binding. The deduced \(K_D\) values are consistent with the ITC measurements.

The catalytic efficiency of the GapB-encoded protein tested with erythrose is highly decreased (\(k_{cat}\) 0.36 s\(^{-1}\), \(K_M\) (erythrose) 90 mM). This points out the role of the phosphate at the C-4 position for revealing the erythrose dehydrogenase activity.

E4P is also a substrate for GAPDH from B. stearothermophilus. \(k_{cat}\) is reduced 760- and 200-fold when compared with that of G3P activity of GAPDH and E4P activity of GapB-encoded protein, respectively (see Table II). This activity is of phosphorylating type as judged by the yield of NADH production.

The NAD Binding to GapB-encoded Protein

To determine whether the higher \(K_M\) value for NAD is indicative of a decrease of its affinity, \(K_D\) value was determined using iodoacetamide as a second order labeling probe of cysteine by measuring the protection against inactivation afforded by the coenzyme binding. The deduced \(K_D\) value provides a measure of macroscopic binding of NAD. Surprisingly, the NAD and NADH dissociation constants \(K_D\) increased 800- and 300-fold, respectively, compared with GAPDH (see Table III).

This can also be related to the observation that GapB-encoded protein is always isolated as an apo form. The low NAD affinity prevented the use of the Racker band (30) as a probe of the active site. ND, not determined. B. st., B. stearothermophilus.

Substituting Asp for Glu-32 did not change drastically \(k_{cat}\) and \(K_M\), values, but allosteric property with a Hill number of 2.3 was revealed with both G3P and E4P substrates (curves not shown) confirming that position 32 is important in revealing cofactor cooperativity in GAPDH (12).
TABLE III

Dissociation constants for NAD and NADH of wild-type GapB-encoded protein and B. steatorrhophilus GAPDH

Inactivation of GapB-encoded protein (5 μM) and B. steatorrhophilus GAPDH (0.2 μM) by iodoacetamide was studied at 37°C in 0.1 M TES buffer, 0.2 mM EDTA, pH 7.3. Kinetics were measured under pseudo first-order conditions, with a 20-fold excess of iodoacetamide (see also “Materials and Methods”).

|                      | GapB-encoded protein | B. steatorrhophilus GAPDH |
|----------------------|----------------------|---------------------------|
| Kᵣ, NAD              | 0.8 ± 0.2            | 1.0 ± 0.1                 |
| Kᵣ, NADH             | 0.14 ± 0.04          | 0.43 ± 0.04               |

DISCUSSION

E. coli strains deleted for gapA gene have been shown to be unable to grow on glucose. This indicated that the high GDP DH activity characterized in E. coli is only encoded by gapA gene and that GapB-encoded protein, if it is translated from its gene, should provide a very low GDP DH activity. The fact that the expressed gapB gene-encoded protein could not suppress the glucose phenotype of a ΔgapA strain supported this conclusion. The kᵣ value of 0.12 s⁻¹ with G3P as a substrate also agreed with this assumption. Indeed, among all the GDP DH mutants from B. steatorrhophilus studied so far, only those having a kᵣ higher than 1 s⁻¹ were shown to complement a ΔgapA E. coli strain (data not shown). The activity of GapB-encoded protein toward G3P is of phosphorylating type and proceeds through a two-step mechanism. First, in the presence of phosphate, formation of 1,3-dPG was observed. Second, 1,3-dPG is a substrate for the reverse reaction. No significant activity is observed when Cys-149 is replaced by a neutral amino acid, whereas replacement of Cys-311 does not abolish the catalytic efficiency of GapB-encoded protein (see “Results”). This supports the role of Cys-149 in the formation of the thioacyl intermediate, as already shown for GDP DH (9). Analysis of the rate constant in the forward direction indicated that the limiting step is association with the formation of the thioacyl intermediate. These steps include G3P binding to the binary complex enzyme-NAD, nucleophilic attack of the Cys-149 on the aldehyde function, hydride transfer, and any potential isomerization step of the ternary complex preceding the hydride transfer. The fact (a) the chemical reactivity of Cys-149 was similar to that of GDP DH and (b) an isotopic effect of 5 was observed when n-[1-²H]G3P was used indicated a limiting step associated with the hydride transfer. Thus, the efficiency of hydride transfer is, at least, a 7000-fold decrease when compared with B. steatorrhophilus GAPDH (14).

Although NAD showed affinity decreased by a factor of 800 when compared with B. steatorrhophilus GAPDH, this does not prevent an efficient acylation step from occurring. Indeed the enzyme showed a low acylation efficiency with G3P, whereas an efficient acylation step with E4P was observed, at least 2000-fold faster. Therefore, the structure of the substrate and its ability to form a productive ternary complex enzyme-NAD-substrate rather prevail, thereby allowing an efficient hydride transfer. The fact that E4P is not an efficient substrate for GDP DH from B. steatorrhophilus (Table II) supports this hypothesis. Different factors can affect the hydride transfer efficiency, in particular the positioning of the hydrogen atom at C-1 relative to the C-4 atom of the nicotinamide ring and/or the positioning of His-176 relative to the OH group at C-1 (the imidazole ring is postulated to play a role as a base catalyst facilitating hydride transfer). Changing His-176 into Asn decreased 40-fold the kᵣ for G3P. Hence, it is tempting to speculate that a non-efficient positioning of the C-1 relative to the pyridinium ring is responsible for the kᵣ decrease. Comparison of the amino acid sequence alignment of the cofactor subunit, i.e., 1-148 and 311-333, indicated that nearly all the residues shown to be involved in the binding of the adenosine (12) and nicotinamide part (11) are conserved, except Asp-32 (see Fig. 1). However, replacing Glu-32 by Asp did not improve the affinity of NAD. Thus other structural factors are involved to explain the drastic increase of Kᵣ. Determination of the three-dimensional structure of GapB-encoded protein in the absence and presence of NAD will help characterize these factors.

Two phosphate anion binding sites, Ps and Pi, were identified in the three-dimensional structure of several GDP DHs (2, 3, 5, 7). The Ps and Pi sites were historically described as those binding the C-3 phosphate of G3P and the inorganic phosphate, respectively (2). The functional role of the two anion binding sites along the catalytic mechanism has not been totally elucidated so far. Modeling studies on glycosomal GDP DH from Leishmania mexicana support G3P binding in the Ps site and an inorganic phosphate binding in the Pi site (5). Although there is an agreement for positioning the inorganic phosphate in the Pi site to attack efficiently the thioester, an alternative mechanism has been proposed for the steps preceding the thioester formation. The C-3 phosphate of G3P would first interact with the Pi site and then flip to the Ps site after the oxidoreduction step had occurred. The structure of the ternary complex GDP DH-glycidol-3-phosphate from B. steatorrhophilus (3) and recent kinetic data obtained from mutants of the Pi and Ps sites favor this mechanism (14, 15). The Ps site is composed of the side chain of Ser-148, Thr-150, Thr-208, and of the peptide NH group of Gly-209, and Thr-179 and Arg-231 contribute to the formation of the Ps site. These amino acids, which are invariant in all the GDP DHs described so far, are also present in GapB-encoded protein except Thr-179 of the Ps site, which is replaced by a Met residue. This substitution could therefore be responsible for the inefficiency of the G3P acylation process by GapB-encoded protein. However, this is unlikely. Indeed, although the T179M mutant of GDP DH from B. steatorrhophilus exhibited a decrease of its kᵣ for G3P by a factor of 400, only the efficiency of the phosphorylating step (and not of the acylation step) was affected (15). Reintroducing a Thr at position 179 did not improve the catalytic efficiency of GapB-encoded protein, in agreement with the prediction. The sequence around the invariant residue Thr-208 of the Ps site (which is located in E. coli GDP DH (7) on a loop between a β-strand and an α-helix) is also largely changed i.e. S206V, S207D, G209K, A210L. In particular, the presence of the side chain of Lys-209 could modify the conformation of the polypeptide main chain around position 208–209 and thus prevent the hydrogen bonding interaction of the main chain of Lys-209 and of the side chain of Thr-208 with inorganic phosphate. However, as for position 179, no improvement of the kᵣ was observed with G3P as a substrate when the sequence of GDP DH of E. coli was reintroduced in GapB-encoded protein. In fact, kᵣ is largely decreased by a factor of 100 (kᵣ = 1.4.10⁻³ s⁻¹ for positions 209–210 and positions 206–207–209–210, respectively), acylation remained rate-limiting. Clearly, although phosphorylation occurs (as proved by the formation of 1,3-dPG) and is more efficient than decylation, we cannot conclude whether or not inorganic phosphate is bound less efficiently to the Ps site of GapB-encoded protein than to the GDP DH site.

How do the above data, which show that GapB-encoded protein is not an efficient phosphorylating GDP DH, fit in with the recent data describing an efficient E4P non-phosphorylating dehydrogenase activity of GapB-encoded protein (23)?

5 F. Della Seta, unpublished results.
First, this new activity raised the question of the nature of the chemical mechanism involved in the oxidation of E4P. Kinetics clearly showed that a two-step mechanism with a limiting step occurring after the acylating step. Molecular modeling of the catalytic domain taking the structure of GAPDH from *E. coli* as a model (see Fig. 7 in Ref. 7) shows an important modification near the catalytic residues Cys-149 and His-176, i.e. Tyr-311, which is conserved in all efficient GAPDHs described so far, is replaced by a Cys residue. Although the presence of the Cys-311 favored the E4P dehydrogenase activity, it is not essential for revealing the activity. Only C149V, A and G mutants lost activity. Clearly, these results support a common chemical mechanism for the oxidation of G3P and E4P catalyzed by GapB-encoded protein with formation of a thiohemiacetal intermediate via Cys-149.

The only structural difference between G3P and E4P is the presence of a supplementary CHOH group in E4P. How is it possible to relate this structural difference to the efficiency of the acylation step that is at least 2000-fold higher for E4P than for G3P? As already mentioned, the binding of E4P to the enzyme-NAD complex is postulated to favor the formation of a ternary complex efficient for the hydride transfer. In the absence of a known three-dimensional structure, it is difficult to define on a structural point of view why E4P and not G3P is capable to favor the formation of an efficient thiohemiacetal ternary complex. The absence of a phosphorylating activity with E4P is also intriguing, while a phosphorylating activity is observed with GAPDH. The binding of inorganic phosphate to the Pi site of the GapB-encoded protein, which should occur as proved by the oxidative phosphorylation of G3P, is not productive for attacking efficiently the thioacyl intermediate formed with E4P. Moreover, $k_{cat}$ is not changed in the presence of a high concentration of phosphate. Although deacylation is not rate-limiting, this supports a binding of inorganic phosphate to the Pi site that does not perturb drastically the positioning and the nucleophilicity of the water molecule attacking the thioacyl intermediate. The rate of deacylation with the E4P substrate for the GapB-encoded protein is higher than 20 s$^{-1}$, whereas the rate of deacylation with the G3P substrate for GAPDH is about 10$^{-2}$ s$^{-1}$ (15). From an energetic point of view, this corresponds to an activation of at least 4.5 kcal·mol$^{-1}$ of the deacylation step. Several kinds of explanations (mutually not exclusive) can be proposed. This energy of activation could correspond to the energy required for the water molecule to attack the thioacyl intermediate. This implies the presence in the active site of the GapB-encoded protein of a base catalyst within the catalytic domain to favor the nucleophilicity of the water molecule. In that context, it would be informative to characterize this amino acid. Another possibility is a more favorable positioning of the thioacyl intermediate toward the attacking water molecule and/or a destabilization of the thioacyl bond within the active site as a consequence of different modes of binding of both substrates. Whatever this issue, it is remarkable to note that GAPDH and GapB-encoded protein, which shares more than 40% of amino acid identity, have evolved from a common ancestor to be efficient as either a phosphorylating or a non-phosphorylating aldehyde dehydrogenase. Moreover, these activities are modulated by the structure of the substrate. This indicates that it could be possible, by a protein engineering approach, to convert a phosphorylating aldehyde dehydrogenase into a non-phosphorylating one and vice versa. Determination of the three-dimensional structure of GapB-encoded protein and of inactive ternary complexes with G3P and E4P will be very instructive in that regard.

**Acknowledgments**—Thanks are due to the Service Commun de Biophysicochimie of the University Henri Poincaré Nancy I for giving us the possibility to realize molecular modeling and to the Service Commun de Résonance Magnétique Nucléaire of the University Henri Poincaré Nancy I for the NMR analyses. We are very grateful to Drs. N. Potier and A. Van Dorsseelaer for determining the molecular weights of wild-type and mutants of GapB-encoded protein and to Dr. M. Hebrant for his technical assistance with stopped-flow experiments. We also thank E. Habermacher and J. P. Decle for their efficient technical help. We thank Drs. B. Charpentier and C. Branlant for providing gapB plasmid constructions.

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