Characterization of the Two Anion-Recognition Sites of Glyceraldehyde-3-phosphate Dehydrogenase from *Bacillus stearothermophilus* by Site-Directed Mutagenesis and Chemical Modification†

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ABSTRACT: The active site of the glycolytic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) contains two anion recognition sites which have been attributed to the phosphate binding of the substrates, namely, glyceraldehyde 3-phosphate (P_s site) and inorganic phosphate (P_i site) [Moras et al. (1975) *J. Biol. Chem. 250*, 9137–9162]. In order to probe the role of both sites during the catalytic event, Arg 195 from the P_i site and Arg 231 from the P_s site of the *Bacillus stearothermophilus* enzyme have been changed to Leu and Gly, respectively, by site-directed mutagenesis. A comparative study of the chemical reactivity of the mutants and wild type toward 2,3-butanedione revealed a similarly high reactivity only for the R195L mutant and wild type, suggesting that only Arg 231 is chemically reactive toward 2,3-butanedione and that its reactivity is not influenced by the presence of the residue Arg 195, which is only 4 Å distant. The kinetic consequences of the mutations were also analyzed for the consecutive steps in the forward catalytic reaction. The replacement of Arg 195 by Leu leads to a marked decrease of the rate of the first steps of the reaction which lead to the acylenzyme formation, in particular, the rate of enzyme–substrate association, while these steps occur at a similar or higher rate when Arg 231 is replaced by Gly. Furthermore, the mutations R195L and R231G also result in a 550-fold and 16 400-fold decrease in the second-order rate constant of phosphorolysis. This step becomes rate-determining for the R195L mutant. These results taken all together favor a reinterpretation of the individual contribution of the P_i and P_s sites [Moras et al. (1975) *J. Biol. Chem. 250*, 9137–9162] during the catalytic event as proposed by Wonacott and co-workers on the basis of *B. stearothermophilus* GAPDH structure [Skarzynski et al. (1987) *J. Mol. Biol. 193*, 171–183]. During the steps preceding the formation of the acylenzyme intermediate, the C-3 phosphate would first interact with the P_i site. The coenzyme exchange step could lead to a conformational isomerization of the acyl group, with the C-3 phophate flipping from the P_i site to the P_s site. Phosphorolysis would then occur by attack of inorganic phosphate with appropriate geometry from the P_i site. The proposed scheme would give a definite functional role to coenzyme exchange.

The glycolytic glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[1] is a tetrameric enzyme which catalyzes reversibly the oxidative phosphorylation of D-glyceraldehyde 3-phosphate (G3P) into 1,3-diphosphoglycerate (1,3-dPG) in the presence of NAD and inorganic phosphate. The currently accepted forward reaction pathway involves two steps: first, an oxidoreduction which leads to the formation of a thioacylenzyme and NADH via a hemithioacetal enzyme intermediate; second, phosphorylation which is favored by the presence of NAD and produces 1,3-dPG (Harris & Waters, 1976).

Two anion-binding sites are present within the active site which were identified from the position of two sulfate ions from the crystallization medium (Buehner et al., 1974; Read et al., 1987; Skarzynski et al., 1987).

On the basis of modeling of the hemithioacetal intermediate to the active site of the lobster GAPDH, the two anion-binding sites were attributed to the binding of C_3-phosphate of the substrate (P_s site) and of the attacking phosphate ion (P_i site) (Moras et al., 1975).

In *Bacillus stearothermophilus* holoenzyme structure (Skarzynski et al., 1987), the P_s site is fully occupied by a sulfate ion which forms hydrogen bonds with the side chains of Arg 231 and Thr 179. It is also hydrogen bonded to the 2′OH of the nicotinamide ribose of NAD (Figure 1). The contribution of NAD seems essential to the formation of this site. Indeed, the P_s site presents a much weaker electron density in the apoenzyme than in the holoenzyme (Leslie & Wonacott, 1984; Skarzynski et al., 1987).

The P_i site in the holoenzyme is only partially occupied by a sulfate ion hydrogen-bonded to the side chains of Ser 148 and Thr 208, the main chain of Gly 209, and the side chain of Arg 195 via two water molecules, providing thus an indirect interaction with the positive charge located on the guanidinium group (Skarzynski et al., 1987).

Numerous studies (McCaul & Byers, 1976; Byers, 1977; Kanchuger et al., 1979) were previously undertaken in order to define the role of the C_3-phosphate of the substrate and in

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[1] Abbreviations: *B. stearothermophilus*, *Bacillus stearothermophilus*; 1,3-dPG, 1,3-diphosphoglycerate; GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; G3P, glyceraldehyde 3-phosphate; *E. coli*, *Escherichia coli*; EDTA, ethylenediaminetetraacetic acid; k_{cat}, catalytic constant; K_M, Michaelis constant; NAD and NADH, nicotinamide adenine dinucleotide (oxidized and reduced form); TES, *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; R195L, Arg 195 → Leu; R231G, Arg 231 → Gly.

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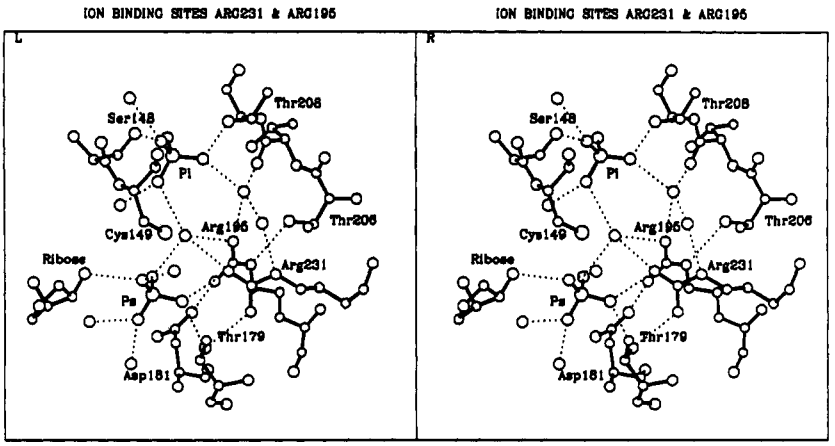


FIGURE 1: Stereoview of the active site of holo-GAPDH from *B. stearothermophilus* showing the ion-binding sites P_s and P_i. The P_s and P_i sites are occupied by sulfate ions in the crystal structure. The conserved residue Arg 231 is hydrogen bonded directly to P_s as is the nicotinamide ribose of NAD. Arg 195 interacts indirectly with P_i through a water molecule. Open circles denote water molecules; hydrogen bonds are shown as dashed lines.

particular to evaluate its contribution to the catalytic efficiency of GAPDH. It was thus concluded that the C_3-phosphate enhances both the reactivity of aldehyde by increasing the stability of the transition state (Byers, 1977) and the selectivity for the D-stereoisomer of G3P (Byers, 1978; Corbier et al., 1989).

However, despite the great number of catalytic (Segal & Boyer, 1953; Duggleby & Dennis, 1974; Meunier & Dalziel, 1978; Canellas & Cleland, 1991; Liu & Huskey, 1992) and energetic studies (Byers, 1977; Kanchuger & Byers, 1979) together with the knowledge of *B. stearothermophilus* holostructure at 1.8-Å resolution, there is no clear evidence as to the individual contribution of P_i and P_s sites during the catalytic cycle.

Moreover, taking into account the greater accuracy of atomic parameters for the *B. stearothermophilus* GAPDH structure, Wonacott and co-workers (Skarzynski et al., 1987) found several structural differences within the active site, pointing out that, "with the revised orientation of the side chain of His176, it was no longer possible to build a reasonable model of hemithioacetal with its C_3-P group in the site designated P_s by Moras et al. (1975)". The authors believed that "although the P_i site must be the location of the inorganic phosphate in the phosphorylation step, it might also play a role in the binding of the C_3-P of G3P". If these assumptions are correct, they imply that the covalent hemithioacetal intermediate must be relocated during the catalytic cycle.

In the present work, Arg 231 and Arg 195 are used as probes to define more precisely the respective role of the P_i and P_s sites during the successive steps of catalysis. For this purpose, mutants R231G and R195L were constructed by site-directed mutagenesis. The kinetic properties of both mutants and their chemical reactivity toward arginyl specific reagent were determined and compared to those of wild-type enzyme. Kinetic arguments are presented suggesting that the C_3-P of the substrate interacts initially with the P_i site during the steps preceding the formation of the acylenzyme intermediate. A revised model of the catalytic event is proposed.

MATERIALS AND METHODS

Site-Directed Mutagenesis, Production, and Purification of Wild-Type and Mutant Enzymes. Construction of recombinant phages and mutagenesis were carried out as previously described by Mougin et al. (1988). Two oligonucleotides of 20-mer and 21-mer were synthesized with one mismatch each at R195 or R231 codons, in order to generate R195L and R231G mutants, respectively.

Production and purification of wild-type and mutant enzymes were performed in *Escherichia coli* W3CG strain (Ganter & Plückthun, 1990) transformed by the recombinant plasmids. Cells were grown in M63 succinate–glycerol medium, containing ampicillin (100 mg/L) and tetracyclin (15 mg/L). After sonication, GAPDH was purified by ammonium sulfate fractionation and loaded on an ACA 34 gel filtration resin equilibrated in 50 mM Tris buffer and 1 mM EDTA, pH 7.0. Final purification was achieved on a Q Sepharose resin by FPLC (Pharmacia).

Apoenzyme was prepared by activated charcoal treatment (Racker & Krimsky, 1952). Protein concentrations were determined spectrophotometrically, using molar extinction coefficients of 1.17×10^5 M^{-1} cm^{-1} and 1.31×10^5 M^{-1} cm^{-1} for *B. stearothermophilus* apo- and holoenzymes, respectively.

Enzyme Activity. Enzyme assays were carried out at 25 °C on a Cary 2200 spectrophotometer by following the appearance or disappearance of NADH at 340 nm. Initial rate measurements were performed according to Ferdinand (1964) in 40 mM triethanolamine buffer, 50 mM K_2HPO_4, and 0.2 mM EDTA at pH 8.9 for the oxidative phosphorylation, and in 10 mM Pipes at pH 7.1 for the reductive dephosphorylation.

Inactivation by 2,3-Butanedione. 2,3-Butanedione concentrations were determined spectrophotometrically using an ϵ_{285} of 25.9 M^{-1} cm^{-1} (Marshall & Cohen, 1980). Wild-type and mutant holoenzymes (1–2 mg/mL) were incubated with 2,3-butanedione (11.5–140 mM) at saturating concentrations of NAD (1 mM) in 100 mM TES buffer and 10 mM EDTA adjusted to pH 7.5 at 22 °C. The inactivation was initiated by addition of small volumes of freshly diluted butanedione. Aliquots were withdrawn at regular intervals and directly diluted in the assay mixture. The remaining activity was measured as described above. As 2,3-butanedione like other dicarbonyl reagents was reported to cause a loss of enzymatic activity due to nonspecific light-induced protein alterations, the inactivation experiments were carried out under dark reaction conditions (Mäkinen et al., 1982). The second-order rate constant (k_2) and the reaction order were calculated according to Scrutton and Utter (1965). Control experiments were performed under identical conditions, except that the modifying agent was omitted and revealed no loss of activity.

Stopped-Flow Kinetic Measurements. Fast kinetic measurements were carried out on a Biologic Instruments (SFM3)

Table 1: Catalytic Parameters of Wild-Type and Mutant GAPDH[a]

	wild-type enzyme		mutant enzyme R195L		mutant enzyme R231G	
	K_M (mM)	k_{cat} (s^{-1})	K_M (mM)	k_{cat} (s^{-1})	K_M (mM)	k_{cat} (s^{-1})
NAD^+	0.15 ± 0.03	70 ± 6	0.14 ± 0.03	6.5 ± 0.3	0.18 ± 0.04	0.15 ± 0.02
G3P	0.80 ± 0.09		1.00 ± 0.10		0.90 ± 0.03	
P_i	8.3 ± 0.6		11.3 ± 1.1		3.7 ± 0.3	
1,3-dPG	0.016 ± 0.004	105 ± 8	0.125 ± 0.014	5.5 ± 0.8	0.102 ± 0.012	0.35 ± 0.04

[a] GAPDH activity was determined at 25 °C in 40 mM triethanolamine buffer, 0.2 mM EDTA, and 50 mM K_2HPO_4 at pH 8.9 for the oxidative phosphorylation and in 10 mM Pipes buffer at pH 7.1 for the reverse reaction. K_M values are the average of three independent determinations.

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-dichloroindophenol by ascorbic acid (Tonomura et al., 1978). 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 340 nm and 25 °C for the GAPDH reaction were directly stored on a Tandom microprocessor. The data were analyzed with the Biokine software using nonlinear regression analysis. An average of at least 5 runs was performed for each determination of rate constants. Unless otherwise indicated, the given concentrations of reactants are those of the final mixture and enzyme concentrations are expressed in sites (N).

(*a*) *Analysis of the Overall Steps Leading to Acylenzyme Formation.* Progress curves of NADH production for wild-type and mutant enzymes were recorded at 340 nm in 50 mM Tris buffer and 2 mM EDTA, pH 8.2, in the absence of inorganic phosphate. One syringe was filled with enzyme (25 μN) and NAD (5 mM), and the other contained G3P (1 mM).

(*b*) *Kinetics of Enzyme–Glyceraldehyde 3-Phosphate Association.* Enzyme–substrate association was performed at high thiol concentration (β-mercaptoethanol: 20 mM) using an approach initially developed by Armstrong and Trentham (1976). In these conditions, the observed pseudo-first-order constant of the transient process was measured for varying G3P concentrations (27.5–220 μM) for wild-type and mutant enzymes.

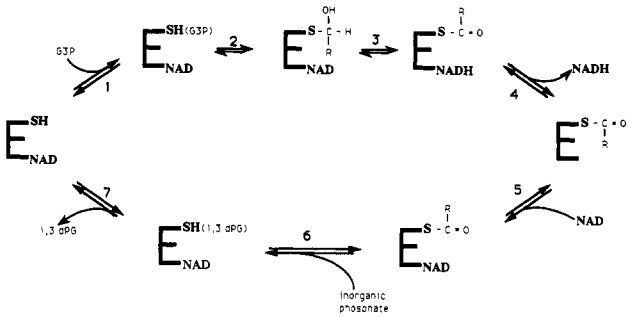
The reaction mixture contained 0.1 M triethanolamine hydrochloride (pH 8.2), 0.2 M NaCl, 1 mM EDTA, 1 mM NAD, 20 mM β-mercaptoethanol, 6 μN enzyme, and 27.5–220 μM G3P. One syringe contained enzyme and NAD, and the other one contained the aldehyde. The other components were present in both syringes.

(*c*) *NADH Dissociation from the Binary Complex GAPDH–NADH.* The rate of NADH dissociation was measured by mixing a solution of GAPDH–NADH binary complex with a trapping solution containing lactate dehydrogenase and pyruvate. As the rate of oxidation by lactate dehydrogenase (350 s^{-1} in our conditions) is higher than the rate of NADH release from GAPDH, the resulting curves could be decomposed into two phases: the first exponential represents the titration of the excess of NADH which is rapidly oxidized by the coupled system, and the second one corresponds to the titration of NADH that dissociates from the binary complex GAPDH–NADH (Clermont et al., 1993).

GAPDH and NADH concentrations were chosen so that at least 3 of the 4 sites were occupied with NADH (as verified by the amplitude of the second phase).

(*d*) *Kinetics of Phosphorolysis.* Phosphorolysis was followed by measuring the steady-state rate of NADH production at low inorganic phosphate concentration so that the observed rate was directly proportional to inorganic phosphate concentration. The reaction was carried out in 50 mM Tris buffer and 2 mM EDTA, pH 8.2. Concentrations of the reactants were 1 mM NAD, 1 mM G3P and 1, 2, and 5 μN for wild-type enzyme and R195L and R231G mutants, respectively. The concentrations for inorganic phosphate varied from 0 to 0.5 mM and from 0 to 2 mM for wild-type enzyme and R195L and R231G mutants, respectively.

Scheme 1: Kinetic Mechanism Proposed by Segal and Boyer (1953)[a]



[a] Steps: **1**, G3P binding to the holoenzyme; **2**, hemithioacetal formation; **3**, hydride transfer; **4**, NADH release; **5**, NAD binding to the acylenzyme; **6**, phosphorolysis; **7**, 1,3-dPG release. R = $CH(OH)CH_2OPO_3^{2-}$.

RESULTS

(*a*) *Steady-State Kinetics.* Steady-state constants are summarized in Table 1. The K_M values of substrates are not changed to any great extent for both mutants apart from an increase of K_M values for 1,3-dPG (6–8-fold). However, both mutants show a significant decrease of k_{cat} values, namely, 10- and 500-fold and 19- and 300-fold less than the wild type, for R195L and R231G mutants in the forward and the reverse reaction, respectively. In order to determine the nature of the limiting steps for both mutants and therefore to define more precisely the role of the P_i and P_s sites during the different steps of the catalytic process, the two distinct chemical steps in the forward reaction, i.e., oxidoreduction (Scheme 1, steps 1–3) and phosphorolysis (Scheme 1, step 6), were separately analyzed at high pH. This study was based on the most currently accepted mechanism for GAPDH (Liu & Huskey, 1992; Trentham, 1971), i.e.: (1) an oxidoreduction step including formation of the thiohemiacetal and hydride transfer (Scheme 1, steps 1–3); (2) NADH release and subsequent replacement by NAD (Scheme 1, steps 4 and 5); (3) acylenzyme phosphorolysis (Scheme 1, step 6).

(*b*) *Consequences of the Mutations on the Overall Steps Leading to Acylenzyme Formation.* The steps leading to acylenzyme formation were studied by transient kinetics in the absence of inorganic phosphate. Under these conditions, deacylation of the acylenzyme becomes rate-determining and thus avoids eventual perturbation of the kinetics due to phosphorolysis.

Table 2: First-Order Rate Constant (k_{obs}) of the Oxidoreduction Reaction Step and Second-Order Constant (k_2) of the Enzyme–Glyceraldehyde 3-Phosphate Association[a]

	wild-type enzyme	R195L mutant	R231G mutant
k_{obs} (s^{-1})	850 ± 150	35 ± 10	975 ± 270
$k_2 \times 10^{-4}$ (M^{-1} s^{-1})	59 ± 1.0	4.6 ± 0.9	640 ± 20

[a] The determination of k_{obs} was performed at 25 °C in 50 mM Tris buffer and 2 mM EDTA adjusted to pH 8.2. The enzyme concentration was 25 μN. The given values are the mean of three determinations carried out on different enzyme preparations. It should be noted that values obtained for wild-type enzyme and mutant enzyme R231G may be slightly underestimated, as the exponential process is very fast and close to the upper limit set by the apparatus (see Materials and Methods). The determination of the second-order constant (k_2) for enzyme–aldehyde association was carried out at 25 °C in 0.1 M triethanolamine buffer, 1 mM EDTA, and 0.2 M NaCl adjusted to pH 8.2. The different concentrations in the final assay were 6 μN enzyme, 2 mM NAD, 20 mM β-mercaptoethanol, and G3P (27.5–220 μM). The value of k_2 was calculated with respect to G3P (total aldehyde) concentration.

For wild-type and mutant enzymes, transient curves of NADH production were obtained. Rate constants (k_{obs}) are representative of the oxidoreduction part of the reaction (Scheme 1, steps 1–3) including G3P binding to holoenzyme, nucleophilic attack of the essential Cys 149, and hydride transfer (and any potential isomerization step of the ternary complex preceding the hydride transfer). The rate constants (Table 2) are in all cases much higher than the k_{cat} values obtained under steady-state conditions (Table 1). Consequently, the rate-determining step occurs after the oxidoreduction step for both mutants and for wild-type enzyme for which the rate-limiting process was associated with NADH release at high pH (Trentham, 1971; Corbier et al., 1990; Clermont et al., 1993)

Moreover, the k_{obs} of the steps leading to acylenzyme formation for the mutant enzyme R231G are similar to that of wild type, while the transient rate constant is reduced at least 25-fold for the mutant enzyme R195L.

The foregoing results suggested a possible participation of Arg 195 in a step preceding hydride transfer and led us to examine the kinetics of association between enzyme and G3P (Scheme 1, step 1). However, difficulties arose from the fact that G3P exists predominantly as its hydrate form (*gem*-diol, 96.6%) and that dehydration leading to the only active form (free aldehyde, 3.4%) is a slow process (k: 0.08 s^{-1}; Trentham et al., 1969; Corbier et al., 1992). As a consequence, at suitable enzyme concentrations required for the stopped-flow experiments, the rate of the reaction tends to be controlled by dehydration of the *gem*-diol form. The use of a "substrate buffer" system initially proposed by Armstrong and Trentham (1976) alleviates this problem, since the hemithioacetal adduct equilibrates rapidly with the unhydrated form of the aldehyde and thus maintains the concentration of free aldehyde constant during the oxidoreduction process. Using this approach, a linear variation of k_{obs} versus aldehyde concentration is observed, up to a concentration of 220 μM of G3P. The values of k_2 (bimolecular rate constant) were calculated with respect to the total aldehyde concentration and are presented in Table 2. It can be seen that the value of k_2 is 11-fold higher for R231G mutant (6.4×10^6 M^{-1} s^{-1}) than for wild-type enzyme (5.9×10^5 M^{-1} s^{-1}) whereas the association rate is slowed 13-fold for R195L mutant (4.6×10^4 M^{-1} s^{-1}). Results from titration experiments carried out under stoichiometric conditions and from molecular modeling (not shown) excluded a productive binding of the *gem*-diol entity (Corbier et al., 1992) within the active site of the wild type and mutants.

Table 3: Dissociation Rate Constant of NADH from the Binary Complex Formed with Wild-Type, R195L, and R231G GAPDHs[a]

enzyme	rate constant k (s^{-1})
wild type	67 ± 5
R195L	52 ± 6
R231G	142 ± 11

[a] A solution of 35 μN lactate dehydrogenase and 10 mM pyruvate was rapidly mixed with an equal volume of NADH (20 μM) and GAPDH (20 μN) for wild-type and mutant enzymes. Both syringes contained 50 mM Tris-HCl buffer and 2 mM EDTA at pH 8.2 with a ionic strength adjusted to 0.15 M by NaCl.

Thus, k_2 is likely to correspond to the association rate constant for free aldehyde. Assuming a 1% free aldehyde concentration under the reaction conditions (Armstrong & Trentham, 1976), a true k_2 value of 6.4×10^8 M^{-1} s^{-1} can be calculated for the mutant R231G. This value is close to the upper limit for a substrate diffusion and is rather high for a substrate like G3P which should be strongly solvated on the C-3 phosphate. This result remains to be explained at the molecular level. The fact that the P_i site is highly solvated (see Figure 1) is an indication that desolvation may not be limiting for the binding of G3P, at least to the wild type.

(*c*) *Rate Constant of NADH Release.* As no suitable relay system is available that would allow determination of the NADH dissociation rate from the acylenzyme intermediate (Clermont et al., 1993), this rate was determined for the binary complex GAPDH–NADH, by using lactate dehydrogenase and pyruvate as a trapping system.

As can be seen in Table 3, NADH dissociates at a rate of 67 s^{-1} for the wild-type enzyme (i.e., at a rate constant similar to the k_{cat} value) and at 52 $s^{-1}$and 142 s^{-1} for R195L and R231G mutants, respectively (i.e., at a rate constant significantly higher than k_{cat}).

The similarity between the NADH dissociation rate from the binary complex GAPDH–NADH and the k_{cat} for the wild-type enzyme—for which the rate-limiting step is assumed to be NADH release from the acylenzyme intermediate (Trentham, 1971; Liu & Huskey, 1992)—strongly suggests that the rate of NADH dissociation is similar for both binary and ternary complexes (Clermont et al., 1993). If the same assumption is valid for the mutant enzymes, this indicates, first, that the rate-limiting step is no longer the coenzyme release for both mutants and, second, that neither Arg 195 nor Arg 231 play a determinant role in facilitating NADH release, as these values are not significantly altered from that obtained for the wild type.

(*d*) *Consequences of the Mutations on the Phosphorolysis Step.* The possibility that phosphorolysis could be rate-determining for both mutants was investigated by comparing the V_M of the reaction in the presence of phosphate and arsenate as acyl acceptor at saturating concentration at pH 8.9 (Canellas & Cleland, 1991). V_M was found to be the same with phosphate and arsenate for wild type and R231G mutant, whereas it was about 2-fold lower for phosphate with mutant enzyme R195L (results not shown), indicating that, in the latter case, phosphorolysis controls the steady-state rate. For R231G, the rate-limiting step is neither NADH release nor phosphorolysis, suggesting that the rate-determining process is related to the coenzyme exchange step.

Nevertheless, the fact that phosphorolysis is not rate-limiting for the mutant enzyme R231G does not exclude a kinetic effect on this step. This was determined by comparing the pseudo-second-order rate constant of phosphorolysis, measuring the rate of the steady state obtained at a concentration of phosphate well below K_M. Under these conditions, the

Table 4: Inactivation of Wild-Type and Mutant Enzymes by 2,3-Butanedione[a]

holoenzyme	n	k_2 (M^{-1} s^{-1})
wild type	1.22 ± 0.03	0.205 ± 0.015
R195L	1.08 ± 0.04	0.079 ± 0.009
R231G	0.39 ± 0.04	0.0013 ± 0.0002

[a] The determinations of the reaction order (n) and the second-order rate constant of inactivation (k_2) were carried out at 22 °C in 0.1 M TES buffer and 10 mM EDTA adjusted to pH 7.5. Holoenzyme concentration for wild-type and mutant enzymes was 1–2 mg/mL in the inactivation assay, and the concentration of 2,3-butanedione was varied from 11.5 to 140 mM. More details are given in the Materials and Methods section. Results given are average values of independent experiments done from three different enzyme preparations.

reaction rate was directly proportional to the phosphate concentration (curves not shown). The values of the second-order rate constants, calculated with respect to inorganic phosphate concentrations, are lowered substantially, from 1.9 × 10^5 M^{-1} s^{-1} for wild-type enzyme to 350 M^{-1} s^{-1} for the R195L mutant and to 11.6 M^{-1} s^{-1} for the R231G mutant.

(*e*) *Reaction Order and Second-Order Rate Constant of the Inactivation by 2,3-Butanedione for Wild-Type and Mutant Enzymes.* In order to investigate the chemical reactivity of the two arginines, 2,3-butanedione was used as a specific chemical reagent. The inactivation of wild-type and mutant holoenzymes by 2,3-butanedione in TES buffer followed pseudo-first-order kinetics and is concentration dependent (curves not shown). The second-order rate constant (k_2) and the reaction order (n) with respect to 2,3-butanedione concentration [I] were deduced from the equation: $\log k' = \log k_2 + n \log [I]$, where k' is the apparent first-order rate constant at a given butanedione concentration. According to Table 4, the reaction orders are 1.22, 1.08, and 0.39, and the second-order rate constants are 0.205 M^{-1} s^{-1}, 0.079 M^{-1} s^{-1} and 0.0013 M^{-1} s^{-1} for the wild type and R195L and R231G mutant enzymes, respectively. Comparison of the data obtained for wild-type and mutant enzymes showed that reactivity is almost the same for wild-type and R195L mutant enzymes, whereas the values obtained for R231G mutant revealed a slow nonspecific inactivation. Therefore, these results strongly suggest a highly selective modification occurring only on the active-site residue Arg 231.

DISCUSSION

Two anion-binding sites labeled the P_i and P_s sites by Rossmann and co-workers are present in the glycolytic GAPDHs which were assigned from the position of two large electron density peaks as sulfate ions from the crystallization medium (Moras et al., 1975). In *B. stearothermophilus* GAPDH, two arginine residues are located within the active sites, namely, Arg 195 and Arg 231 (Figure 1), which are associated with the P_i and P_s sites, respectively (Skarzynski et al., 1987). Residue Arg 231 is invariant in all known glycolytic GAPDH primary structures while Arg 195 is only present in GAPDH isolated from thermophilic organisms. The hypothesis that the invariant Arg 194 residue in mesophilic GAPDHs could be the structural equivalent of Arg195, and not of the Arg 194 which is also present in thermophilic GAPDHs, as a result of a deletion at position 189 in thermophilic GAPDHs (Skarzynski et al., 1987) has been recently discarded on the basis of the knowledge of the crystal structure of *E. coli* GAPDH at high resolution (Dideberg, personal communication). A complete superposition of the Arg 194 side chain of *E. coli* and *B. stearothermophilus* GAPDHs is observed, excluding any possible contribution of Arg 194 to the formation of the P_i site in mesophilic GAPDHs.

Using an arginine-specific chemical reagent, holo R195L mutant is shown to have a second-order rate constant and a reaction order toward 2,3-butanedione similar to that observed for wild type while holo R231G mutant is 160- and 60-fold less reactive toward 2,3-butanedione than wild type and R195L mutant, respectively. Therefore, these results strongly support a specific modification of residue Arg 231 with a high chemical reactivity toward 2,3-butanedione. Moreover, although Arg 195 contributes to catalytic efficiency (see Table 1), this residue can be considered to be essentially chemically unreactive toward 2,3-butanedione. This study also indicates that the chemical reactivity of Arg 231 is not significantly influenced by the proximity of Arg 195, which is less than 4 Å away in the holoenzyme of *B. stearothermophilus* (Skarzynski et al., 1987).

The free energy relationship studies carried out by Byers and co-workers to examine the nature of the interaction of various aldehyde substrates including G3P with GAPDH indicated a more effective interaction of the C_3-phosphate in the transition state of the rate-limiting step than in the ground state (Kanchuger et al., 1979). Clearly, both mutations studied here affect k_{cat} rather than K_M in the forward reaction. However, K_M and k_{cat} values are both significantly altered in the reverse reaction. These apparently contradictory results lead us to investigate more precisely the effects of both mutations at the level of the individual steps of catalysis in the forward reaction. Although the limiting step for both mutations is shown to occur after the oxidoreduction step (see Results, section c), this could not exclude a change in the rate of the steps preceding or concomitant with the hydride transfer, namely, the association step between holo GAPDH and G3P, a putative isomerization step leading to a productive ternary complex (Corbier et al., 1990) or hydride transfer.

Clearly, the rate of NADH formation is slowed overall only for the R195L mutant by a factor of 24. On the other hand, a 13-fold decrease of the second-order rate constant of GAPDH–aldehyde association was observed for the R195L mutant while the R231G mutant exhibited a rate of enzyme–substrate association higher than that of wild type. The change in the kinetics of G3P association for the R195L mutant could be explained by a poorer interaction of the C_3-phosphate of G3P within the altered active site, resulting in a less favorable positioning of G3P relative to the essential Cys 149 and leading to a less productive formation of the hemithioacetal intermediate compared to that of wild type and the R231G mutant. Taken together, these results suggest that Arg 195 which forms part of the so-called P_i site is involved in the initial interaction with the C_3-phosphate of G3P, leading to the formation of the hemithioacetal intermediate while Arg 231 associated with the P_s site does not play a significant role in binding the C_3-phosphate of G3P. This is supported by the crystal structure of a ternary complex *B. stearothermophilus* holo-GAPDH–glycidol 3-phosphate which revealed a binding of the C_3-phosphate of the inhibitor to the P_i site (Skarzynski et al., 1987).

With the aim of assessing the role of Arg 231 and Arg 195, and bearing in mind the hypothesis of Grau et al. (1982) that the primary effect of a charge repulsion betwen His 176 and Arg 231 could facilitate NADH release, we have also examined a possible contribution of these residues to NADH dissociation. Our experimental results show a rate of NADH release for R231G and R195L mutants similar to that of wild type, indicating that Arg 231 and Arg 195 play no role in this step

of the reaction (see Table 3). This finding is in agreement with the study of Nagradova and co-workers on rabbit muscle and yeast GAPDHs showing no contribution of Arg residues to acylenzyme–NADH complex destabilization (Asryants et al., 1989). Moreover, these results support a limiting step for both mutants occurring after the release of NADH from the ternary complex acylenzyme intermediate–NADH.

A kinetic investigation of the phosphorolysis step shows that the second-order rate constant is markedly decreased by a factor of 550 and 16 400 for mutants R195L and R231G, respectively. Assuming the previous conclusion that the formation of the thiohemiacetal intermediate implies a binding of the C_3-phosphate of G3P to the P_i site, two possible schemes could be envisaged for the last steps of the forward reaction (Scheme 1, steps 4–6). In the first one, C_3-phosphate would remain bound to the P_i site during the phosphorylating step, implying that inorganic phosphate should bind to the P_s site. In the second, a conformational isomerization step would occur preceding the phosphorylating step, with the C_3-phosphate of the acylenzyme intermediate flipping from the P_i site to the P_s site, leading to the binding of inorganic phosphate to the P_i site. This isomerization step would take place during coenzyme exchange, during which the P_s site becomes more accessible (Skarzynski et al., 1987).

Although we cannot settle between the two hypotheses, two arguments support the latter. First, kinetic studies show that the phosphorylation becomes rate-determining only for the R195L mutant. In this case, the decrease of the rate of phosphorylation could be due to a loss of an efficient interaction between the inorganic phosphate and the mutated P_i site, which in turn could alter the rate of phosphorolysis. Second, model building of the intermediates of the reaction based on the refined structure of the *B. stearothermophilus* GAPDH strongly suggests that the appropriate geometry for the inorganic phosphate attack at the C-1 of the acylenzyme intermediate is only satisfied from the P_i site (Skarzynski et al., 1987). For the R231G mutant, the rate-limiting step is neither phosphorolysis nor NADH release but is rather associated to a conformational isomerization preceding the phosphorylating step. This result is not in contradiction with the substantial decrease of the rate observed for the phosphorylating step. An altered P_s site could also destabilize the binding of the acylenzyme to the P_s site, which in turn can lead to an inefficient attack of inorganic phosphate from the P_i site.

The proposed conformational isomerization of the acylenzyme intermediate would also give a definite functional role to coenzyme exchange. The replacement of NADH by NAD could facilitate the conformational isomerization through the positive charge of the nicotinamidium ring. A crucial point in favor of a kinetic scheme in which NADH release and subsequent NAD binding precede phosphorolysis is that the presence of NAD greatly enhances the rate of phosphorolysis (Trentham, 1971). From an energetic point of view, the free energy level of 1,3-dPG is higher than that of the corresponding thioester (Bücher, 1947; Jencks et al., 1960) so that about 6 kcal are required for phosphorylating the acylenzyme intermediate. This implies that the thioester needs to be destabilized and the presence of NAD, presumably via the positive charge of the nicotinamidium, could be a determining factor.

Determination of the three-dimensional structure of stable acylenzyme and noncovalent ternary complexes would shed more light on the catalytic mechanism for phosphorylating GAPDH.

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