# A New Chemical Mechanism Catalyzed by a Mutated Aldehyde Dehydrogenase†

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ABSTRACT: NAD(P) aldehyde dehydrogenases (EC 1.2.1.3) are a family of enzymes that oxidize a wide variety of aldehydes into acid or activated acid compounds. Using site-directed mutagenesis, the essential nucleophilic Cys149 in the NAD-dependent phosphorylating glyceraldehyde-3-phosphate dehydrogenase from *Escherichia coli* has been replaced by alanine. Not unexpectedly, the resulting mutant no longer shows any oxidoreduction phosphorylating activity. The same mutation, however, endows the enzyme with a novel oxidoreduction nonphosphorylating activity, converting glyceraldehyde 3-phosphate into 3-phosphoglycerate. Our study further provides evidence for an alternative mechanism in which the true substrate is the gem–diol entity instead of the aldehyde form. This implies that no acylenzyme intermediate is formed during the catalytic event. Therefore, the mutant C149A is a new enzyme which catalyzes a distinct reaction with a chemical mechanism different from that of its parent phosphorylating glyceraldehyde-3-phosphate dehydrogenase. This finding demonstrates the possibility of an alternative route for the chemical reaction catalyzed by classical nonphosphorylating aldehyde dehydrogenases.

The NAD(P)[1] aldehyde dehydrogenases are a class of enzymes which catalyze the oxidation of a wide variety of aldehyde substrates (Blackwell et al., 1989). This reaction consists of a hydride ion transfer occurring from the aldehydic carbon of the substrate to the C-4 position of the nicotinamidium of the oxidized coenzyme. From a chemical point of view, the enzymatic oxidation of aldehyde to acid or activated acid is facilitated if the hydride ion transfer is first preceded by a conversion of the aldehyde into a tetrahedral intermediate (Fersht, 1984). Good evidence derived from biochemical studies shows that the tetrahedral intermediate is formed within the active site of the enzyme via the nucleophilic attack by an essential amino acid on the aldehydic group of the substrate. The acylenzyme intermediate formed during the oxidation step is then attacked either by a water molecule in the nonphosphorylating aldehyde dehydrogenases or by inorganic phosphate in phosphorylating aldehyde dehydrogenases to produce acid or activated acid, respectively. In both cases, the essential nucleophilic amino acid present within the active site is assumed to be a cysteine residue (Harris & Waters, 1976; Hempel et al., 1982, 1991; Von Bahr-Lindström et al., 1985; Tu & Weiner, 1988; Kitson et al., 1991; Blatter et al., 1992).

The present paper describes a mutant of the phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Escherichia coli* where the nucleophilic essential cysteine has been replaced by an alanine residue. Biochemical evidence is provided to show that the mutant GAPDH is a new enzyme which uses a chemical mechanism different from that of the parent enzyme.

## MATERIALS AND METHODS

(*a*) *Mutagenesis and Isolation of Enzyme.* Site-directed mutagenesis, production, and purification of wild-type and mutant *E. coli* enzymes were performed as already described (Mougin et al., 1988). No SH group was titratable for the mutant by 5,5′-dithiobis(2-nitrobenzoate) under nondenaturating conditions, confirming the absence of Cys149 in the mutant protein.

(*b*) *Enzyme Assays.* Initial rate measurements were carried out at 25 °C on a Cary 2200 spectrophotometer by following the absorbance of NADH at 340 nm. For the forward reaction, the experimental conditions were 40 mM triethanolamine, 2mM EDTA, 50 mM $K_2HPO_4$, pH 8.9 (Ferdinand, 1964). For the reverse reaction, assays were performed in 10 mM PIPES, pH 7. Turnover number ($k_{cat}$) was calculated using an $\epsilon_{280} = 1.24 \times 10^5$ $M^{-1}$ $cm^{-1}$ for the tetramer (Mougin et al., 1988) and was expressed per site.

The initial rate data were fitted to the Michaelis–Menten relationship using least-squares analysis to determine $V_M$ and $K_M$. All $K_M$ values were determined at saturating concentrations of the other substrates. Inhibition constants ($K_i$) were determined by Dixon plots.

(*c*) *Enzymatic Characterization of the Reaction Products.* HPLC analysis of the reaction products was made as follows: 25 μmol of glyceraldehyde 3-phosphate (G3P), 5 μmol of NAD, and 10 μmol (40 μmol in sites) of GAPDH were incubated at room temperature in 0.1 M $K_2HPO_4$, pH 7.6, in a total volume of 5 mL. In the case of wild-type enzyme, a coupled system (20 μmol of lactate dehydrogenase, 0.3 mmol of pyruvate) was added in order to displace the equilibrium of the reaction. This system was not used in the case of the mutant enzyme, since the reaction is irreversible. Enzymes were removed by centrifugation after denaturation by addition of $H_2SO_4$ (pH 2.5). The clear solution was diluted with water to 20 mL and applied onto a DEAE column (LKB 5PW) equilibrated in 25 mM imidazole buffer, pH 7. Elution was performed with a linear gradient of KCl (0 → 0.5 M).

The content of the fractions was finally tested enzymatically; 1,3-diphosphoglycerate (1,3-dPG) was characterized using the reverse reaction of GAPDH (see above) whereas 3-phos-

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[1] Abbreviations: GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; G3P, glyceraldehyde 3-phosphate; 3-PG, 3-phosphoglycerate; 1,3-dPG, 1,3-diphosphoglycerate; $P_i$, inorganic phosphate; NAD and NADH, nicotinamide adenine dinucleotide (oxidized and reduced form); *B. stearothermophilus*, *Bacillus stearothermophilus*; *E. coli*, *Escherichia coli*; C149A, Cys149 → Ala; C149S, Cys149 → Ser; C149G, Cys149 → Gly.

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phoglycerate (3-PG) was characterized enzymatically using a coupled system with phosphoglycerate kinase (Vas & Batke, 1990).

(*d*) *Kinetic Characterization of the Enzymatically Active Form of G3P.* The determination of the enzymatically active form of G3P was performed by stopped-flow kinetics using a Biologic Instrument interfaced with a Tandom PC ASL 486-110 computer. The data were analyzed with the Biokine software. Reactions were initiated by mixing the enzyme (6–24 $\mu$N and 50–200 $\mu$N in sites for the wild type and the mutant C149A, respectively) and NAD (1 mM) from one drive syringe and G3P (0.5–2 mM and 0.6–2 mM for the wild type and the mutant C149A, respectively). Both syringes contained 200 mM triethanolamine, pH 8.6; $Na_2HAsO_4$ (30 mM) was also added in the case of the wild type. The concentration of G3P was expressed in terms of D-G3P, which is the active form of the substrate.

## RESULTS AND DISCUSSION

Glyceraldehyde-3-phosphate dehydrogenases belong to the aldehyde dehydrogenase family and can be either phosphorylating or nonphosphorylating. The nonphosphorylating GAPDH catalyzes the irreversible oxidation of G3P into 3-PG (Kelly & Gibbs, 1973; Pupillo & Faggiani, 1979). Relatively little is known about the chemical mechanism of this type of GAPDH except from chemical modification experiments on the enzymes from *Chlamydomonas reinhardtii* (Iglesias et al., 1987) and from spinach leaves (Iglesias & Losada, 1988) which suggest the direct implication of an essential cysteine residue. The phosphorylating GAPDH, where the essential catalytic residue is Cys149, catalyzes the reversible oxidation of G3P into 1,3-dPG. Clearly, the nucleophilic property of the sulfur group of Cys149 ought to be essential for oxidative phosphorylation. Accordingly, substitution of the Cys149 residue by a neutral amino acid is expected to abolish any phosphorylating activity.

This hypothesis was tested by constructing the C149A mutant of *E. coli* GAPDH by site-directed mutagenesis. The choice for this substitution was dictated by steric considerations based on the high-resolution structure of the enzyme from *Bacillus stearothermophilus* (Skarzynski et al., 1987; Skarzynski & Wonacott, 1988). Surprisingly, the classical enzymatic assay of GAPDH, as monitored by the conversion of the NAD into NADH in the forward reaction, reveals that the C149A enzyme remains active. However, the pH–$k_{cat}$ profile of the mutant C149A shows a sigmoidal behavior with an apparent p$K_a$ of 6.6 different from the value of 7.4 determined for the wild type (Figure 1). Moreover, this activity is the same whether or not phosphate ions are present, indicating that phosphate is no longer a substrate. The same is true for arsenate, an alternative acyl acceptor. Furthermore, analysis of the reverse reaction shows no reductive dephosphorylation activity with 1,3-dPG (Table I). Clearly, the mutated enzyme exhibits an activity different from that of its parent phosphorylating GAPDH.

The product formed in the forward reaction is therefore expected to be not 1,3-dPG but 3-PG. Its chemical nature was established by means of enzymatic analysis after isolation by HPLC (see Materials and Methods).

The unexpected activity observed for the C149A mutant raises the question of the nature of the chemical mechanism of catalysis. One possibility is that another nucleophilic amino acid [see, e.g., Loomes et al. (1990)] in the phosphorylating GAPDH active site could play the role ascribed to Cys149. This hypothesis is, however, rather unlikely. First, from a kinetic point of view, the formation of a hypothetical acylenzyme intermediate for the C149A mutant implies that the rate of water hydrolysis of this intermediate will be at least equal to the steady-state turnover, i.e., 5 $s^{-1}$. However, the rates of hydrolysis of the acylenzyme intermediate for the wild type and the C149S mutant at pH 8.9 were determined to be 0.01 and 0.001 $s^{-1}$, respectively (Corbier et al., unpublished experiments). Therefore, the efficiency of $H_2O$ hydrolysis catalyzed by the mutated enzyme would have to be at least 500- and 5000-fold higher than that of the wild type and the C149S mutant, respectively. Moreover, the fact that only 3-PG is produced implies that the hypothetical acylenzyme intermediate formed during the oxidative process catalyzed by the C149A mutant should be efficiently hydrolyzed but not phosphorylated, although the $P_i$ site is still present (Skarzynski et al., 1987; Skarzynski & Wonacott, 1988; Branlant & Branlant, 1985). From a structural point of view, such mechanistic schemes seem rather dubious. Second, in contrast to the wild type (Soukri et al., 1989) and the mutant C149S (Corbier et al., unpublished results), the mutant C149A does not show esterolytic activity against *p*-nitrophenyl acetate, which argues against the presence of a reactive nucleophilic residue within the active site of the C149A mutant.

Hence, the absence of an active site nucleophilic residue in the mutant C149A that could covalently bind the substrate

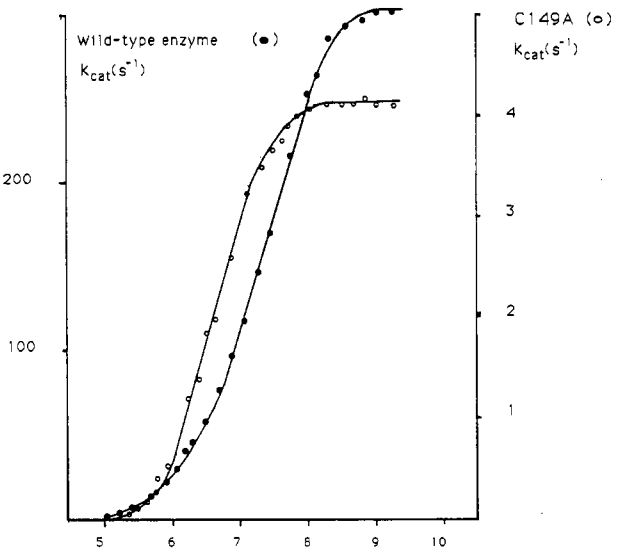


FIGURE 1: Variation of the catalytic rate constant with pH. The pH dependence of the overall rate of the reaction was studied in varying buffers to ensure a constant ionic strength, close to 0.1 M. Buffers: pH 5.0–6.2, 180 mM acetate, 70 mM EDTA, 50 mM $K_2HPO_4$ buffer; pH 6.0–7.5, 100 mM imidazole, 5 mM EDTA, 50 mM $K_2HPO_4$ buffer; pH 7–9.5, 40 mM triethanolamine, 0.2 mM EDTA, 50 mM $K_2HPO_4$ buffer. Symbols: (●) GAPDH, *E. coli* wild-type enzyme; (○) GAPDH, *E. coli* C149A mutant enzyme.

Table I: Kinetic Parameters of Wild-Type and Mutant C149A *E. coli* GAPDH[a]

| | wild-type enzyme | C149A mutant |
|---|---|---|
| $k_{cat}$ ($s^{-1}$) | 264 ± 32 | 5.2 ± 0.6 |
| $K_M$ G3P (mM) | 1.5 ± 0.2 | 3.6 ● 0.5 |
| $K_M$ NAD (mM) | 0.042 ± 0.005 | 0.074 ● 0.004 |
| $K_M$ $P_i$ (mM) | 22 ± 4 | not substrate |
| $K_M$ 1,3-dPG (mM) | 0.015 ● 0.004 | not substrate |
| $K_I$ 1,3-dPG (mM) | 0.011 ● 0.003 | 0.014 ± 0.003 |

[a] GAPDH activity was determined as described in Materials and Methods. Over the wide range of concentration tested for phosphate and arsenate (0–200 mM) no variation of activity was detected at constant ionic strength ($I$ = 0.2 M) for the C149A mutant enzyme, suggesting that neither phosphate nor arsenate can act as acyl acceptor.

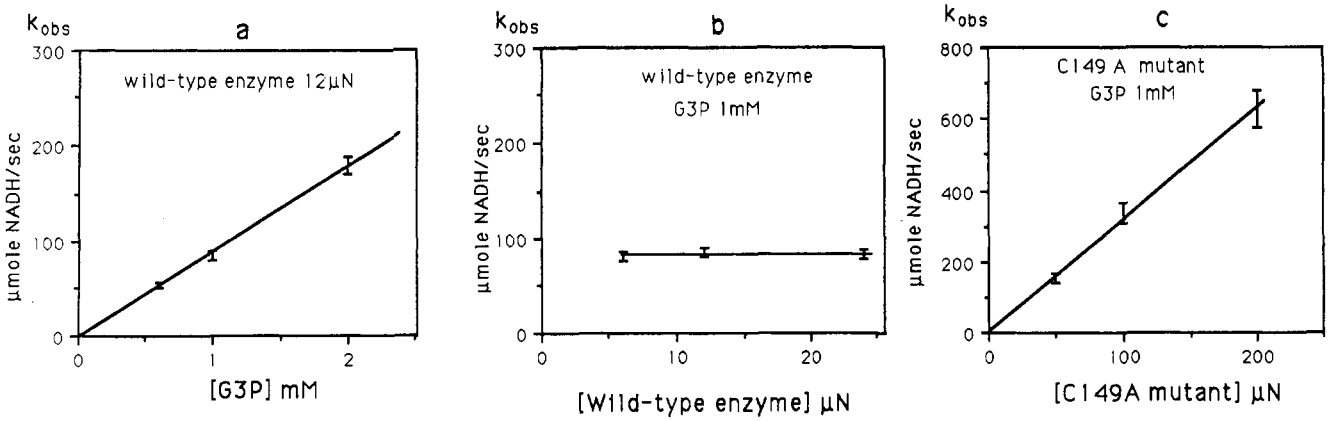


FIGURE 2: Kinetic characterization of the enzymatically active form of G3P used by the *E. coli* C149A mutant enzyme. The experimental conditions are those described in Materials and Methods. For wild type, the yield of NADH produced in the pre-steady-state phase corresponded to the concentration of free aldehyde (curves not shown). As shown in part a, the steady-state rate is directly proportional to G3P concentration and independent of the high concentration of enzyme used (part b). So under these conditions, the rate of conversion of diol into aldehyde is the rate-limiting process as already shown by Trentham (Trentham et al., 1969). The rate of the steady-state ($k_{obs}$) provides a direct measure of the interconversion rate ($k$) of gem-diol to aldehyde with $k_{obs} = k$[G3P gem-diol]. The value of $k$ is calculated on the basis of two sets of three independent experiments (mean value of at least 4 traces each) and is equal to $0.085 \pm 0.004$ $s^{-1}$ in good agreement with that found by Trentham (Trentham et al., 1969). For the mutant C149A, no burst of NADH appearance was observed (curve not shown). For the three concentrations of G3P tested (0.6–2 mM), the steady-state rate of NADH production is found to be directly proportional to the high concentration of enzyme used (part c) and higher than that observed for wild type where the conversion of diol into aldehyde is the rate-limiting process (part b).

during catalysis supports a mechanism involving a gem-diol entity as the reacting substrate. The hypothesis that a gem-diol entity could be the form of substrate dehydrogenated by pyridine dinucleotide dependent dehydrogenases has already been postulated for glyoxylate oxidation catalyzed by lactate dehydrogenase (Warren, 1970) but is yet not proven before the present study. Only arguments derived from structure–activity relationship studies carried out with alcohol dehydrogenase from *Drosophila melanogaster* have suggested that such a mechanism could be biologically operative (Eisses, 1989). In solution, the aldehyde form of G3P is in equilibrium with the hydrate form in a molar ratio of 1:29 at pH 8.6 (Trentham et al., 1969). In the case of the wild-type GAPDH, where the aldehyde species is the true substrate, the limiting step in catalysis depends on the rate constant for conversion of the predominant gem-diol form of G3P to the aldehyde form at high enzyme concentration (Trentham et al., 1969). Yet, we show that the steady-state rate of NADH production at high concentrations of C149A mutant is greater than that predicted if the interconversion rate had been rate limiting (Figure 2). This finding hence further argues for the gem-diol entity acting as the true substrate.

The activity of the C149A mutant implies favorable positioning of the gem-diol substrate within the active site of the *E. coli* mutant, in particular in relation to the pyridinium ring of NAD and to His176 for efficient hydride transfer (Soukri et al., 1989). This positioning should be similar to that proposed for the thiohemiacetal intermediate (Moras et al., 1975; Biesecker et al., 1977), with one of the two hydroxyl groups of the gem-diol entity in place of the thiol group of Cys149 and the other in a suitable position for deprotonation by the imidazole of His176. In this way, the hydrogen atom on C-1 is pointed directly at the C-4 atom of the nicotinamidium. However, the presence of the side chain of Ala appears essential for the productive positioning of the gem-diol within the active site. Indeed, no measurable activity is detected with mutant C149G. Preliminary X-ray data of the C149G mutant from *B. stearothermophilus* suggest that while the native structure of this mutant is maintained, the substrate appears to be bound in a nonproductive mode (Wonacott, personal communication). Therefore, a rather precise structural conformation of the active site cavity seems an absolute requirement for the catalytically productive positioning of gem-diol substrate.

The value of $k_{cat}$ of the mutant enzyme is significant, i.e., 2/100 of the wild type, and is similar to that of the nonphosphorylating GAPDH (Iglesias & Losada, 1988) and in the same range as generally described for aldehyde dehydrogenases (Blackwell et al., 1987; Hill et al., 1991; Bhatnagar et al., 1991). From an evolutionary point of view, the question still remains why the chemical mechanism involving the gem-diol entity as the active form of aldehyde substrates is not biologically operative for nonphosphorylating aldehyde dehydrogenases. Determination of the three-dimensional structure of nonphosphorylating aldehyde dehydrogenases at high resolution, in particular that of nonphosphorylating GAPDH, should shed more light on this issue.

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