Determinants of Coenzyme Specificity in Glyceraldehyde-3-phosphate Dehydrogenase: Role of the Acidic Residue in the Fingerprint Region of the Nucleotide Binding Fold†

S. Clermont,‡ C. Corbier,‡ Y. Mely,§ D. Gerard,§ A. Wonacott,∥ and G. Branlant*,‡

Laboratoire d'Enzymologie et de Génie Génétique, Université de Nancy I, URA CNRS 457, BP 239, 54506 Vandoeuvre-lès-Nancy Cédex, France, Laboratoire de Biophysique, Faculté de Pharmacie, URA CNRS 491, 74 Route du Rhin, BP 24, 67401 Illkirch Graffenstaden Cedex, France, and Protein Structure Group, Glaxo Group Research Limited, Greenford Road, Greenford, Middlesex UB6 OHE, U.K.

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ABSTRACT: On the basis of the three-dimensional structure of the glycolytic NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and of sequence comparison with the photosynthetic NAD(P)-dependent GAPDH of the chloroplast, a series of mutants of GAPDH from *Bacillus stearothermophilus* have been constructed. The results deduced from kinetic and binding studies suggest that the absence of activity of the wild-type GAPDH with NADP as a cofactor is the consequence of at least three factors: (1) steric hindrance, (2) electrostatic repulsion between the charged carboxyl group of Asp32 and the 2′PO_4, and (3) structural determinants at the subunit interface of the tetramer. The best value for k_{cat}/K_M and K_D for NADP was observed for the D32A-L187A-P188S mutant. This triple mutation leads to a switch in favor of NADP specificity but with a k_{cat}/K_M ratio 50- and 80-fold less than that observed for the wild type with NAD and for the chloroplast GAPDH with NADP, respectively. Substituting the invariant chloroplastic Thr33-Gly34-Gly35 for the *B. stearothermophilus* Leu33-Thr34-Asp35 residues on the double mutant Ala187-Ser188 does not improve significantly the affinity for NADP while substituting Ala32 for Asp32 on the double mutant does. Clearly, other subtle adjustments in the adenosine subsite are needed to reconcile the presence of the carboxylate group of Asp32 and the 2′-phosphate of NADP. Kinetic studies indicate a change of the rate-limiting step for the mutants. This could be the consequence of an incomplete apo–holo transition. The results taken all together suggest that it is difficult from a rationale to predict all the structural determinants that are implicated in determining specificity for a coenzyme. Comparative studies of the binding properties of various mutants suggest an essential role of position 32 and more generally of the adenosine subsite for revealing negative or positive cooperativity in GAPDH. Furthermore, it is suggested that a correct positioning of the pyridinium ring is necessary but not sufficient for inducing cooperativity.

A number of proteins including NAD(P)[1] dehydrogenases, kinases, or aminoacyl transfer RNA synthetases exhibit common structural features known as the Rossmann fold domain (Rossmann et al., 1975). This nucleotide binding domain consists of an α,β structure with a central parallel β sheet flanked on each side by a layer of α helices. Sequence analysis led Wierenga et al. (1985, 1986) to propose the existence of a fingerprint of 11 specific positions in NAD dehydrogenases including (see Table I) (a) an invariant GXGXXG sequence which extends from the C-terminal end of the first β strand to the N-terminal end of the following α helix, (b) a hydrophobic core composed of six small residues, and (c) an acidic residue, usually Asp, at the C-terminus of the second β strand, which hydrogen bonds to the hydroxyl groups of the adenine ribose. Although most NAD-linked dehydrogenases possess this fingerprint structure, some contain small variations (Wierenga et al., 1986; Bork & Grunwald, 1990; Teller et al., 1992).

A consensus sequence has also been proposed for NADP binding proteins in which the third glycine of the NAD fingerprint is replaced by an alanine (Hanukoglu & Gutfinger, 1989) and which usually includes a positively charged residue in the neighborhood of the C-terminus of the $\beta\alpha\beta$ motif. Again this fingerprint is not unequivocally correlated with NADP specificity (Benachenhou & Baldacci, 1991; Loeber et al., 1991; Teller et al., 1992; Kumar et al., 1992; Stelandre et al., 1992). The role of a positively charged residue would be to confer specificity for NADP and to discriminate against NAD by interacting specifically with adenosine 2′-phosphate. Thus the C-terminal region of the second β strand seems to be crucial in determining specificity for coenzyme and controlling the relative affinity of dehydrogenases for NAD and NADP. Several recent studies argue in favor of this interpretation. First, the substitution of the invariant Asp residue of three different NAD dehydrogenases, namely, lactate dehydrogenase (Feeney et al., 1990) and alcohol dehydrogenases from

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* To whom correspondence should be addressed.
‡ Laboratoire d'Enzymologie et de Génie Génétique.
§ Laboratoire de Biophysique.
∥ Glaxo Group Research Limited.

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[1] Abbreviations: GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; NAD and NADH, nicotinamide adenine dinucleotide (oxidized and reduced form); NADP and NADPH, nicotinamide adenine dinucleotide phosphate (oxidized and reduced form); *B. stearothermophilus*, *Bacillus stearothermophilus*; *E. coli*, *Escherichia coli*; 3-CAPAD, 3-(chloroacetyl)pyridine adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; Tris, *N*-tris(hydroxymethyl)aminomethane; D32A, Asp32 → Ala; D32N, Asp32 → Asn; D32E, Asp32 → Glu; S, Leu187 → Ala-Pro188 → Ser; B, Leu33 → Thr-Thr34 → Gly-Asp35 → Gly; K_M, Michaelis constant; k_{cat}, catalytic constant; P_i, inorganic phosphate; G3P, glyceraldehyde 3-phosphate.

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Table I: Sequence Alignment of NAD-Dependent *B. stearothermophilus* and Dual NAD(P)-Dependent Chloroplastic GAPDHs[a]

coenzyme specificity	source	1 10 20 30 40
NAD	Bacillus stearothermophilus	AVKVGINGFG RIGRNVFRAA LKN---PDIEVVA VNDLTDANT- L
		ββββββ αααααααααααα ββββββββ ααααα
NAD(P)	Nicotiana tabacum (gene A)	KLKVAINGFG RIGRNFLRCW HGR-KDSPLDVIA INDTGGVKQ- A
NAD(P)	Zea mays (gene A)	KLKVAINGFG RIGRNFLRCW HGRGDASPLDVIA INDTGGVKQ- A
NAD(P)	Zea mays (pseudogene)	KLKVAINGFG RIGRNFLRCW HGRGDDSPLDVIA INDTGGVKQ- A
NAD(P)	Pisum sativum (gene A)	KQLKVAINGFG RIGRNFLRCW HGR-KDSPLDVIA INDTGGVKQ- A
NAD(P)	Spinacia oleracea (gene A)	KLKVAINGFG RIGRNFLRCW HGR-KDSPLDVVV INDTGGVKQ- A
NAD(P)	Arabidopsis thaliana (gene A)	KLKVAINGFG RIGRNFLRCW HGR-KDSPLDIIA INDTGGVKQ- A
NAD(P)	Nicotiana tabacum (gene B)	KLKVAINGFG RIGRNFLRCW HGR-KDSPLDVVV VNDSGGVKN- A
NAD(P)	Pisum sativum (gene B)	KLKVAINGFG RIGRNFLRCW HGR-KDSPLEVIV VNDSGGVKN- A
NAD(P)	Spinacia oleracea (gene B)	KLKVAINGFG RIGRNFLRCW HGR-KDSPLDVVV VNDSGGVKS- A
NAD(P)	Arabidopsis thaliana (gene B)	KLKVAINGFG RIGRNFLRCW HGR-KDSPLEVVV LNDSGGVKN- A

[a] The numbering fo the amino acid sequence was according to Biesecker et al. (1977). The consensus amino acids which sign the $\beta\alpha\beta$ fold in NAD-dependent GAPDHs are illustrated in boldface. The secondary structure of the $\beta\alpha\beta$ fold is indicated under the *B. stearothermophilus* GAPDH sequence on the basis of its crystalline structure (β, β strand; α, α helix; nothing, loop). References for sequences are *B. stearothermophilus*, Branlant et al. (1989); *Nicotiana tabacum*, Shih et al. (1986); *Zea mays*, Quigley et al. (1988–1989); *Pisum sativum*, Brinkmann et al. (1989) and Liaud et al. (1990); *Spinacia oleracea*, Brinkmann et al. (1989) and Ferri et al. (1990); *Arabidopsis thialiana*, Shih et al. (1991).

Drosophila (Chen et al., 1991) and yeast (Fan et al., 1991) with serine, asparagine, and glycine, respectively, led to mutants catalytically more efficient with NADP and with equal or lower efficiency with NAD than the wild-type enzymes. Second, the mutation of Arg198 of NADPH-specific glutathione reductase to Met substantially decreased activity with NADPH, while it increased the activity with NADH (Scrutton et al., 1990). However, six further mutations within the $\beta\alpha\beta$ unit were necessary to create a glutathione reductase that used NADH substantially more efficiently than NADPH. Thus, the presence of negatively or positively charged residues is probably a key factor contributing to the relative specificity of NAD or NADP as cofactor, but other factors including steric and conformational constraints are certainly also involved. This is the case for the tetrameric NAD-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Bacillus stearothermophilus* where two substitutions located outside the $\beta\alpha\beta$ fold structure but close to the adenosine ribose of the *R*-axis-related subunit produced a mutant that is active with NADP as a cofactor (Corbier et al., 1990a). Similarly, the absence of activity with Asp38→Leu and Asp38→Arg mutants from *Drosophila* alcohol dehydrogenase could be interpreted in part as a consequence of steric hindrance which in turn perturbs the conformation of the active site (Chen et al., 1991).

No high-resolution three-dimensional structures are yet available for dehydrogenases that exhibit dual-coenzyme specificity. However, sequence alignment of dual-coenzyme dehydrogenases such as glutamate dehydrogenase (Teller et al., 1992, and references cited therein) and GAPDH (Table I) reveals the presence of structural features specific of a $\beta\alpha\beta$ fold with a consensus sequence GXGXXG and an acidic residue at the C-terminus of the second β strand. This signature had previously been regarded as indicative of specificity for NAD.

GAPDH is a good model for addressing this question. First, the three-dimensional structures of both the holo and apo forms of GAPDH from *B. stearothermophilus* have been determined at high resolution and contain the ADP binding fingerprint typical of NAD-dependent dehydrogenases with the invariant Asp residue at position 32, at the C-terminus of the second β strand (Skarzynski et al., 1987; Skarzynski & Wonacott, 1988). Second, GAPDH from chloroplast shows dual-coenzyme specificity, with a preference for NADP[2] and is homologous to *B. stearothermophilus* GAPDH with 55% sequence identity (Martin & Cerff, 1986). Third, replacement of amino acids Leu187 and Pro188 of GAPDH from *B. stearothermophilus* by Ala and Ser (herein referred to as S) from the homologous chloroplast GADPH conferred activity with NADP (Corbier et al., 1990a).

In order to investigate the contribution of Asp32 to coenzyme specificity in GADPH from *B. stearothermophilus*, a series of mutants have been constructed in which Asp32 has been replaced. These have been combined with other mutations around the adenosine binding pocket, including the S mutant. This paper describes the enzymatic and cofactor binding properties of these mutants.

MATERIALS AND METHODS

(*a*) *Mutagenesis and Isolation of Mutated Enzymes.* Site-directed mutagenesis and production and purification of wild-type and mutant *B. stearothermophilus* enzymes were performed as described earlier (Mougin et al., 1988). GAPDH concentrations were determined spectrophotometrically, using a molar extinction coefficient of 1.17×10^{-5} M^{-1} cm^{-1} and 1.31×10^{-5} M^{-1} cm^{-1} for the apo- and holoenzymes, respectively. Enzyme concentrations are expressed either in molarity (M), i.e., tetramer concentration, or in normality (N), i.e., subunit concentration.

(*b*) *Enzyme Assays.* Assays were performed with a Cary or a Kontron Uvikon spectrophotometer, following the appearance of NAD(P)H at 340 nm. Initial rate measurements were carried out in 40 mM triethanolamine buffer, 0.2 mM EDTA, and 50 mM K_2HPO_4, pH 8.9 (Ferdinand, 1964). Apparent Michaelis constants were determined at the saturation concentration of inorganic phosphate (P_i). Glyceraldehyde 3-phosphate (G3P) concentrations were chosen to allow maximum reaction rate while avoiding substrate inhibition.

[2] Little kinetic data are so far available for the chloroplastic GAPDHs. Cerff (1978) reported results for chloroplast GAPDH from *Sinapis alba* showing that NAD and NADP were competitive inhibitors of NADPH in the reductive dephosphorylation step with K_i constants of 1.2 and 0.048 mM, respectively. Ferri et al. (1978) determined the following constants on spinach chloroplast GAPDH: for NAD, $k_{cat} = 22$ s^{-1}, $K_M = 0.2$ mM, and $k_{cat}/K_M = 110$ s^{-1} mM^{-1}; for NADP, $k_{cat} = 47$ s^{-1}, $K_M = 0.04$ mM, and $k_{cat}/K_M = 1175$ s^{-1} mM^{-1}.

Michaelis constants were deduced from nonlinear regression data analysis.

(*c*) *Inactivation of GAPDH by 3-(Chloroacetyl)pyridine Adenine Dinucleotide (3-CAPAD).* Experimental conditions were the same as those described previously (Corbier et al., 1990a,b) except that TES was used instead of phosphate buffer. The presence of phosphate ion was shown to decrease drastically the inactivation kinetic constant and the binding affinity of NADP. This could be due to the occupancy of the two anion binding sites of GAPDH by phosphate ion, which in turn would decrease the alkylation rate of Cys149, and to a competition between phosphate and the 2′-phosphate of NADP, which would increase the apparent K_D constant of NADP.

(*d*) *Spectrofluorometric Measurements.* Determination of NAD(P) binding affinities at 22 °C was performed as previously described for the *Escherichia coli* enzyme (Corbier et al., 1990b). In short, quenching of tryptophan fluorescence by NAD(P) was used to monitor the binding of NAD(P) to both wild-type and mutant enzymes. Fluorescence measurements were performed with a Perkin-Elmer MPF 66 spectrofluorometer. Excitation and emission wavelengths were set at 295 or 298 and 330 or 350 nm, respectively. Fluorescence intensities were corrected for the screening effect due to the absorption of NAD(P) and for Raman emission by subtracting the buffer emission spectrum.

Making the reasonable assumption that the binding of NAD(P) to each enzyme subunit gave an identical fluorescence decrease, the average number ν of moles of NAD(P) bound per mole of enzyme could be obtained using $\nu = n(I_F - I_{FO})/(I_{FT} - I_{FO})$, where I_F is the fluorescence measured for any added NAD(P) concentration while I_{FO} and I_{FT} correspond to the fluorescence intensities of the apoprotein and the holoenzyme [with its n coenzyme binding sites saturated with NAD(P)], respectively. The concentration x of free NAD(P) was calculated using $x = [NAD(P)]_t - n[E]_t$, where $[NAD(P)]_t$ and $[E]_t$ represent respectively the total NAD(P) and enzyme concentrations. Finally, ν and x were fitted to the Adair–Klotz equation by a nonlinear least-squares procedure allowing the determination of the macroscopic dissociation constants K_i:

$$\nu = \frac{\frac{x}{K_1} + \frac{2x^2}{K_1K_2} + \ldots + \frac{nx^n}{K_1K_2\ldots K_n}}{1 + \frac{x}{K_1} + \frac{x^2}{K_1K_2} + \ldots + \frac{x^n}{K_1K_2\ldots K_n}}$$

In the case of low-affinity species, it was necessary to use high NAD(P) concentrations which led to the appearance of an additional quenching of fluorescence probably due to a nonspecific binding of NAD(P) to the enzyme. This additional fluorescence decrease was also observed for high-affinity species in the presence of a high molar excess of NAD(P) and was shown to vary linearly with the NAD(P) concentration. Thus the fluorescence F associated with the saturation of the specific coenzyme binding sites could be easily computed from the measured fluorescence F_m by $F = F_m + a[NAD(P)]$, where a is the slope deduced from the experiments made with the high-affinity species. All calculations and curve fitting were carried out using SAS computer procedures.

Due to the low accuracy of the K_3 and K_4 determinations, the cooperative behavior of the subunits of the GAPDH to coenzyme binding was estimated from the ratio of macroscopic dissociation constants K_2/K_1. Only a K_2/K_1 ratio significantly greater than 2.7 is indicative of anticooperativity. Indeed, if we consider a system where all n subunits are identical and independent with a microscopic dissociation constant k, macroscopic constants are calculated using $K_i = i/(n - i + 1)k$. As a consequence, if $n = 4$, anticooperative behavior will only be significant if $K_2/K_1 > 2.7$.

(*e*) *Stopped-Flow Kinetic Measurements.* Fast kinetic measurements were carried out on a Biologic Instruments (SFM3) stopped-flow apparatus with a tungsten lamp as light source. The continuously measured absorbance was digitized and directly stored on a Tandon microprocessor. An average of at least five runs was performed for each determination of the rate constants. Data were analyzed with the Biokine software using nonlinear regression analysis. The dead time of the apparatus (under the conditions employed) was shown to be 1.4 ms by measuring the reduction of 2,6-dichlorophenolindophenol by ascorbic acid (Tonomura et al., 1978).

Analysis of the Rate of the First Turnover of the Reaction. In the absence of P_i, acyl enzyme hydrolysis becomes rate limiting, allowing the measurement of the rate of NAD(P)H production during the first turnover of the reaction (Corbier et al., 1990b). Progress curves for the reaction of wild-type and mutant enzymes with G3P and NAD(P) were recorded at 340 nm and 25 °C. The concentrations of the reactants were 20 μN enzyme, 1 mM G3P, and 5 mM NAD(P). Both syringes contained 50 mM Tris and 2 mM EDTA buffer, pH 8.

Determination of the NAD(P)H Dissociation Rate from the Binary Complex GAPDH–NAD(P)H. The rate of NADH dissociation from GAPDH was measured by mixing a solution of the GAPDH–NADH binary complex with a trapping NADH solution containing lactate dehydrogenase and pyruvate. The decrease in NADH concentration was monitored by the decrease of absorbance at 340 nm. If the rate of NADH oxidation by lactate dehydrogenase (390 s^{-1} in our experimental conditions) is higher than the rate of NADH releasing from GAPDH, then the resulting progress curves could be decomposed into two exponentials: the first one will represent the titration of the excess of free NADH that is rapidly oxidized by the coupled system (k = 390 s^{-1}), and the second one will correspond to the titration of NADH that dissociates from the binary complex GAPDH–NADH. A similar approach was used to determine the rate of NADPH dissociation from mutants: in this case, the trapping solution contained glutathione reductase (the rate of NAPDH oxidation is 310 s^{-1} in our experimental conditions) and oxidized glutathione. In both experiments, GAPDH and NAD(P)H concentrations were adjusted so that at least three of the four sites were occupied with NAD(P)H (as checked by the amplitude of the second phase).

RESULTS

Justification of Mutations. Choice of amino acid substitutions was based on the complementary approaches of sequence comparison (see Table I) around residues 33–40 and molecular modeling (Corbier et al., 1990a, and discussion therein).

Steady-State Kinetic Analysis of Wild Type and Mutants. The kinetic parameters for wild type and various mutants are summarized in Table II. All the mutants have increased apparent Michaelis constants (K_M) for NAD with the following order: wild type < S < D32A < D32A–S = B–S < D32E < D32N; all except the D32E mutant revealed activity with NADP. A quite different order is observed for the apparent K_M value for NADP: D32A–S < B–S < D32N < S = D32A. No correlation is observed between the variation of K_M and k_{cat} values. Generally, the k_{cat}/K_M ratio is considered to be the most meaningful measure of enzyme substrate specificity

Table II: Kinetic Parameters of Wild-Type and Mutant GAPDHs[a]

	NAD			NADP		
	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})
wild type	0.1	70	700		ND[c]	
D32E	0.9	61	68		ND	
D32A	0.5	70	140	7.1	2	0.3
S[b]	0.4	70	175	7.1	15	2
D32N	2.8	22	8	3.6	14	4
B–S	0.8	80	100	2.7	18	7
D32A–S	0.8	7	9	1.3	18	14

[a] The reaction was performed at 22 °C in 40 mM triethanolamine, 50 mM K_2HPO_4, and 0.2 mM EDTA buffer, pH 8.9. Each value is the average of at least three determinations. The standard deviation is below 20%. [b] From Corbier et al. (1990a). [c] ND, activity not detectable.

(Fersht, 1985). From this point of view, all mutations have a large effect on NAD specificity, especially for D32N and D32A–S mutants. Nevertheless, D32A–S mutation provides an enzyme more specific for NADP than NAD, although catalytic efficiency with NADP is 50-fold smaller than that of the wild type with NAD.

Effect of the Mutations on the Rate-Limiting Step. The fact that catalytic parameters k_{cat} and apparent K_M with NAD(P) were modified by mutations raised the question whether the rate-limiting step is the same for the wild type (Trentham, 1971a,b; Corbier et al., 1990b) and mutants and thus whatever the coenzyme used.

Two dual-coenzyme-specific mutants were chosen to address these questions: the S mutant, more specific for NAD than NADP (with k_{cat}/K_M values of 175 and 2, respectively), and the D32A–S mutant, more specific for NADP than NAD (k_{cat}/K_M values of 14 and 9, respectively).

In the absence of P_i, at pH higher than 8, fast kinetic experiments showed a burst of NAD(P)H production at a rate of 700–800 s^{-1} for both mutants, similar to that observed with the wild type with NAD as a coenzyme (curves not shown). These results indicate that the rate-limiting step process takes place after the hydride-transfer step. Therefore, the limiting step could only be associated with either (a) NAD(P)H release from the acyl enzyme intermediate, (b) NAD(P) binding to the acyl enzyme intermediate, or (c) the phosphorylating step. The fact that the maximal velocity was not dependent on the chemical nature of the acyl enzyme acceptor, i.e., phosphate or arsenate, excluded hypothesis c (data not shown). Hypothesis a was tested using an electron chemical [phenazine methosulfate and 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide; Abdallah & Biellmann, 1980] or enzymatic (cytochrome *c* reductase) relay system. Unfortunately, both systems were shown to oxidize NAD(P)H far too slowly to allow determination of the NAD(P)H dissociation rate (data not shown).

Thus, only the NAD(P)H dissociation rate from binary complexes GAPDH–NAD(P)H was determined using lactate dehydrogenase and glutathione reductase as NADH and NADPH trapping systems, respectively. This approach was used successfully for malate dehydrogenase (Lodola et al., 1978). As indicated in Table III, the NADH dissociation rate from wild-type GAPDH is similar to the k_{cat} value, i.e., 70 s^{-1}. If the limiting step is assumed to be NADH release from the acyl enzyme intermediate as already postulated (Trentham, 1971a,b; Liu & Huskey, 1992), this would indicate a similar rate of NADH release for both binary and ternary complexes.

As shown in Table III, coenzyme dissociation rate constants for S and D32A–S mutants are estimated to 117 and 298 s^{-1} for NADH and 226 and 133 s^{-1} for NADPH, respectively. These rates are greater than the k_{cat} values, i.e., 70 and 7 s^{-1} for NADH and 15 and 18 s^{-1} for NADPH. Assuming the same hypothesis as for the wild type, such results suggest that the limiting rate for both mutants would not be NAD(P)H release from the acyl enzyme intermediate and argue in favor of a limiting step associated with NAD(P) binding (which could include a limiting conformational transition step) to the acyl enzyme entity.

Table III: Dissociation Rate Constant of NAD(P)H from the Binary Complex Formed with Wild-Type, S, and D32A–S GAPDHs[a]

	NADH (s^{-1})	NADPH (s^{-1})
wild type	67	ND[b]
S	117	226
D32A–S	298	133

[a] For NADH dissociation rate determination, a solution of 60 μN lactate dehydrogenase and 20 mM pyruvate was rapidly mixed with an equal volume of NADH and GAPDH, 30 μM and 30 μN for wild type, 80 μM and 40 μN for the S mutant, and 40 μM and 40 μN for the D32A–S mutant, respectively. The rate of oxidation of free NADH by lactate dehydrogenase was shown to be 390 s^{-1}. For NADPH dissociation rate determination, a solution of 50 μN glutathione reductase and 4 mM oxidized glutathione was mixed with an equal volume of 50 μM NADPH and 30 μN S or D32A–S mutants. The rate of oxidation of free NADPH by glutathione reductase was shown to be 310 s^{-1}. Both syringes contained 50 mM Tris and 2 mM EDTA buffer, pH 8.2, with the ionic strength adjusted to 0.15 M. In the same buffer, in the presence of 50 mM K_2HPO_4, the k_{cat} value of the wild type is 70 s^{-1}. The standard deviation is below 5%. [b] ND, not determined.

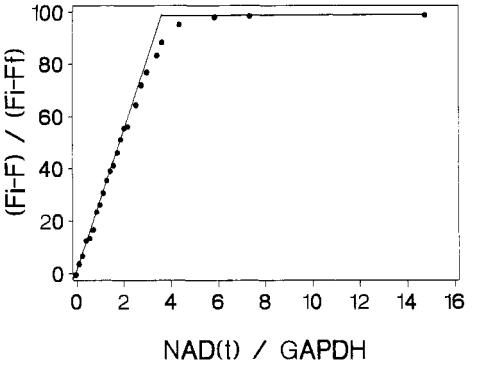

FIGURE 1: NAD binding curve of wild-type GAPDH. The enzyme concentration was 13.5 μM in 100 mM Tris–0.2 mM EDTA, pH 8. The fluorescence intensity F monitored at 330 nm was expressed as $(F_i-F)/(F_i-F_f)$, where F_i and F_f represent the fluorescence intensity in the absence of NAD and in the presence of a great molar excess of NAD, respectively. Typically the F_f/F_i ratio is about 0.63 for wild-type and various mutant GAPDHs.

Binding of NAD(P) to Wild-Type and Mutant GAPDHs. To determine whether K_M values for NAD(P) could be considered as indicative of the affinity constant for the different mutants tested, two additional experimental approaches were taken.

First, the binding of NAD(P) was studied by measuring the quenching of the GAPDH tryptophan fluorescence intensity observed upon coenzyme binding. The *B. stearothermophilus* GAPDH contains two Trp residues at positions 84 and 310 (Branlant et al., 1989). Absorption and fluorescence spectra of native and mutant GAPDHs were similar (curves not shown), and the maximum emission wavelength of native and mutant enzymes was 330 nm. Due to its positioning within the active site, Trp310 is believed to be the highest emitting fluorescent probe on the basis of phosphorescence studies (Gabellieri & Strambini, 1989). As shown in Figure 1, the binding of NAD to the wild-type enzyme under our experimental conditions was stoichiometric with a

Table IV: NAD(P) Macroscopic Dissociation Constants to Wild-Type and Mutant GAPDHs[a]

	K_1 (μM)	K_2 (μM)	K_3 (μM)	K_4 (μM)	$\bar{K}$ (μM)	K_2/K_1
			(A) Macroscopic Dissociation Constants of NAD			
wild type	0.04 ± 0.01	0.19 ± 0.02	0.45 ± 0.2	1.3 ± 0.6	*0.26 ± 0.07*	4.7 ± 0.65
S	0.30 ± 0.06	0.8 ± 0.2	1.2 ± 0.1	19 ± 1	*1.5 ± 0.2*	2.7 ± 1.2
D32A	0.27 ± 0.08	1.0 ± 0.1	0.6 ± 0.2	500 ± 200	*3.0 ± 0.8*	3.7 ± 1
B–S	3.5 ± 1	13 ± 2	800 ± 700	800 ± 700	*70 ± 30*	3.7 ± 0.6
D32N	19 ± 3	31 ± 2	180 ± 30	1400 ± 200	*110 ± 10*	1.6 ± 0.2
D32A–S	40 ± 20	300 ± 100	1700 ± 900	420 ± 90	*300 ± 100*	7.5 ± 0.6
D32E	130 ± 30	5.1 ± 0.8	50 ± 10	300 ± 100	*60 ± 10*	0.04 ± 0.01
			(B) Macroscopic Dissociation Constants of NADP			
wild type	180 ± 10	360 ± 80	1400 ± 900	400 ± 100	*440 ± 90*	2 ± 0.5
S	40 ± 20	150 ± 70	2000 ± 1000	400 ± 200	*250 ± 100*	3.8 ± 3.6
D32A	70 ± 40	390 ± 20	800 ± 500	440 ± 90	*310 ± 80*	5.6 ± 0.9
B–S	30 ± 5	80 ± 10	3000 ± 1000	700 ± 300	*270 ± 60*	2.7 ± 0.5
D32N	67 ± 9	150 ± 30	200 ± 100	700 ± 200	*190 ± 50*	2.2 ± 0.4
D32A–S	1.4 ± 0.4	10 ± 5	350 ± 40	1700 ± 300	*55 ± 10*	7.1 ± 5.6

[a] Binding experiments and data analysis were performed as indicated in Materials and Methods. Dissociation constants are expressed as means ± standard deviation. The dissociation constants above 200 μM are only indicative. The dissociation constants of NADP to the D32E mutant are not given because of too low affinities. $\bar{K}$ is the overall macroscopic dissociation constant given by $\bar{K} = (K_1K_2K_3K_4)^{1/4}$.

plateau for one NAD per site. Moreover, in these conditions the fluorescence decrease was clearly linear, validating the assumption that the binding of NAD to each enzyme subunit induced the same fluorescence decrease. Due to limitation in protein material and experimental conditions, very low binding constants (K_i > 200 μM) could not be evaluated accurately. For these reasons, the low K_3 and K_4 binding constants of several mutants for NAD(P), and the resulting overall macroscopic dissociation constant $\bar{K}$, should be taken only as indicative. Therefore, cooperative properties will be discussed on the basis of the K_1 and K_2 values and the corresponding ratio K_2/K_1. The results obtained for mutant GAPDHs are summarized in Table IV [NAD(P) binding to D32E is not considered]. Thus, K_1 and K_2 constants for NAD binding increased by factors up to 1000 and 1580, respectively, with the following binding sequence: wild type < S ≃ D32A ≪ B–S < D32N < D32A–S. The order for NADP binding is almost the reverse, except for D32N, K_1 and K_2 values decreasing by factors of up to 130 and 36, respectively. Clearly, the D32A–S mutation provides the best affinity for NADP. Apart from the D32E mutant, the other mutants have a K_2/K_1 ratio around 2.7. This indicates that they exhibit no cooperativity or slight anticooperative behavior with either NAD or NADP at least for the first two subunits. Nevertheless, it appears that position 32 is of crucial importance to cooperativity with NAD. While wild type and D32A mutants show slight anticooperativity, D32N abolishes it whereas the D32E mutation provides strong positive cooperativity.

As further indication of coenzyme binding, the dissociation constant K_D for NAD(P) was determined using 3-CAPAD as an affinity-labeling probe by measuring the protection against inactivation afforded by the coenzyme binding (Corbier et al., 1990a,b; Branlant et al., 1982). The deduced K_D value provides a measure of macroscopic binding and overcomes the experimental limitations of the fluorescence quenching at high concentrations of coenzyme. The NAD and NADP dissociation constants K_D for the three mutants, D32A, S, and D32A–S (Table V), varied in a manner similar to that for K_1, K_2, or $\bar{K}$ determined by fluorescence studies, indicating a consistent interpretation of the experimental data. It is important to note that the constants, i.e., K_1, K_2, $\bar{K}$, K_D, and K_M, tend to show the same trends.

The K_D values of 3-CAPAD itself were also determined and are shown to vary in the same way as with NAD, not surprising since 3-CAPAD is a coenzyme analogue of NAD which is chemically modified on the pyridinium ring (Biellmann et al., 1973). The magnitude of the rate constant for the alkylation (k) is probably determined primarily by the position of the chloromethyl group relative to Cys149. For wild-type and mutated GAPDHs, k values are closely similar, showing that, prior to inactivation, the orientation of the chloromethyl group and the Cys149 side chain is similar in the wild-type and mutant enzyme binary complexes.

Table V: Dissociation Constants of 3-CAPAD, NAD, and NADP to Wild-Type and Mutant GAPDHs from *B. stearothermophilus* As Determined by Affinity Labeling with 3-CAPAD[a]

	k (min^{-1})	K_D(3-CAPAD) (μM)	K_D(NAD) (μM)	K_D(NADP) (μM)
wild type	3.5	22	0.9	35
D32A	3.2	28	0.6	57
S	2.5	25	1.1	36
D32A–S	2.8	423	126	3.4

[a] Inactivation of apo wild-type (0.8 μN), D32A (1.2 μN), S (0.8 μN), and D32A–S (5 μN) mutants was studied at 22 °C in 0.1 M TES and 0.2 mM EDTA buffer, pH 8.4. The concentrations of 3-CAPAD used were a function of the K_D values (between 0.5 and 5 of the K_D value). In all cases, the 3-CAPAD inactivation process followed pseudo-first-order kinetics. Inactivation rate constants (k) and dissociation constants (K_D) of 3-CAPAD were determined from curves representing the double-reciprocal plot of the apparent inactivation rate k_{app} versus 3-CAPAD concentration. NAD(P) dissociation constants were determined from plots of k_{app}^{-1} versus NAD(P) concentration at a fixed concentration of 3-CAPAD, i.e., 12, 23, 58, and 106 μM for the wild-type, D32A, S, and D32A–S mutants, respectively. The resulting curves were interpreted according to Kitz and Wilson (1969), Meloche (1967), and Branlant et al. (1982).

DISCUSSION

Analysis of the structural differences observed between the apo and holo-GAPDH structures has led to the proposal of a mechanism which accounts for the conformational changes associated with NAD binding (Skarzynski & Wonacott, 1988). According to this mechanism, the ADP moiety of NAD can first bind to GAPDH in the apo conformation. Domain closure, which is postulated to be required for productive binding of the nicotinamide moiety, would also generate improved interactions at the adenosine subsite. The holo structure model shows that the acidic residue Asp32 is involved in the specific binding of the adenosine ribose through hydrogen bonds with both hydroxyl groups of the ring. Moreover, the carboxylate group is also held in place by hydrogen bonds from the main-chain NH groups of residues 8 and 33 situated

at the carboxy-terminal ends of the adjacent strands of the β sheet that form the $\beta\alpha\beta$ motif of the ADP binding fold (Skarzynski & Wonacott, 1988).

The absence of activity with GAPDH from *B. stearothermophilus* with NADP as a cofactor was previously postulated to be the consequence of at least three factors (Corbier et al., 1990a): (1) steric hindrance; the activity of the S mutant from *B. stearothermophilus* with NADP supported this hypothesis (Corbier et al., 1990a); (2) the presence of the charged carboxyl group as Asp32 which would generate strong electrostatic repulsion of the 2′-PO_4; (3) the absence of hydrophilic amino acids at the adenosine subsite whose role would be to stabilize the binding of NADP by hydrogen bonds with the 2′-PO_4.

Our results clearly show that steric factors are indeed one of the principal determinants of coenzyme specificity. Replacing Leu187-Pro188 by Ala-Ser (S mutation) in the D32A mutant results in a significant increase of the affinity for NADP. In contrast, D32A or S mutations give only low affinity for NADP. For the D32A–S and D32A mutants, we found a 50- and 40-fold decrease in the K_1 and K_2 values, respectively, while factors of only 2.5 for K_1 and 1 for K_2 are observed between wild type and the D32A mutant. These results support our previous molecular modeling (Corbier et al., 1990a) which showed that NADP could fit the coenzyme binding pocket of the holoenzyme only if steric hindrance is relieved for the 2′-PO_4 group. On the contrary, NAD shows the opposite behavior; that is, the more space that is available, the weaker its binding. Thus, replacing Leu187-Pro188 by Ala-Ser in the D32A mutant results in a drastic reduction in the affinity for NAD. For the S and D32A–S mutants we observe a 130- and 380-fold decrease in the K_1 and K_2 values, respectively, whereas only a factor of 7.5 for K_1 and 4 for K_2 is observed between wild type and the S mutant. Mutational effects are qualitatively additive, but their magnitude is not readily predicted. This is illustrated well by the behavior of the D32A–S mutant which binds NADP efficiently but NAD poorly while D32A and S mutants individually bind NAD quite well but NADP weakly (see Tables II, IV, and V).

An electrostatic repulsive contribution also intervenes in determining the cofactor specificity of GAPDH. Recent crystallographic studies of the S mutant at pH 6.9 showed that the 2′-PO_4 of NADP is positioned outside the adenosine subsite so as to minimize unfavorable interaction with Asp32, which is presumed to remain negatively charged. Only a single hydrogen bond between Asp32–OD1 and NADP–O3′ atoms remains (Wonacott, unpublished observations). Our binding and kinetic studies done at pH higher than 8 are in agreement with these results. First, no NADP activity is detected with the D32E mutant, while good activity is still observed with NAD. Second, dual-coenzyme specificity is observed with the D32N mutant where replacement of Asp32 by Asn could be considered as a conservative mutation with a steric bulk largely unaltered. Here both cofactors bind to the mutant but with a low affinity, which suggests that the Asp residue is present as a carboxylate, with its negative charge localized within the adenosine subsite.

Our results partly contradict the fact that the Asp32 residue is invariant in all chloroplast GAPDHs which are dual-coenzyme specific. Our previous molecular modeling study (Corbier et al., 1990a) assigned a possible specific role to the invariant residues Thr33 (chloroplastic gene A) or Ser33 (chloroplastic gene B), Gly34, and Gly35, which are Leu, Thr, and Asp, respectively, in the GAPDH from *B. stearothermophilus* (Corbier et al., 1990a) (Table I). On the one hand, the presence of Thr or Ser at position 33 is expected to increase the hydrophilicity of the adenosine subsite while the presence of residues Gly34 and Gly35 would give the peptide loop 32–36 more flexibility and hence potential space for the 2′-PO_4 of NADP. The combined mutations, i.e., L33T-T34G-D35G (B mutations) carried out on the S mutant, however, do not improve significantly the affinity for NADP while substituting Ala32 for Asp32 on the double mutant does. Other subtle adjustments in the adenosine subsite are therefore needed to reconcile the presence of the carboxylate group of Asp32 and the 2′-phosphate of NADP. The conversion of an NAD glycolytic GAPDH into an efficient chloroplastic GAPDH[2] in which an aspartate residue is retained at position 32 requires further investigation.

Recent studies based on structural considerations and sequence homology in both NADP and dual-specific glutamate dehydrogenases, most of which possess a conserved acidic residue located at the C-terminus of the $\beta\alpha\beta$ motif, have led Baker et al. (1992) to suggest the existence of two distinct classes of NADP dehydrogenases which could be differentiated by their preference for either the monobasic or dibasic 2′-phosphate. In the case of the monobasic form, one of the phosphate oxygen atoms is protonated and could act as a hydrogen bond donor to the carboxyl group of Asp. Dual-specific GAPDH and glutamate dehydrogenase are suggested to belong to the latter class (Baker et al., 1992). An NMR titration study of the 2′-PO_4 of NADP (Ehrlich & Colman, 1992) bound to chloroplast GAPDH and a comparative study with dual-coenzyme-specific mutant GAPDHs would be very informative in the above regard.

Analysis of the different kinetic steps suggests that the limiting step for the wild type is associated with NADH release from the acyl enzyme intermediate as already postulated by Trentham (1971a,b). This behavior seems to be different for the S and D32A–S mutants. The limiting rate is most likely associated to the NAD(P) binding to the acyl enzyme intermediate which could lead to a partial apo–holo transition of the ternary complex. Recent crystallographic studies carried out on the binary complex S mutant–NADP support this interpretation. A rotation of the coenzyme domain relative to the catalytic domain of only 1.5° (Wonacott, unpublished observation) was observed instead of 4.3° observed for NAD binding to the wild type (Skarzynski & Wonacott, 1988). (However, the full apo–holo transition is not precluded during the formation of the active ternary complex of GAPDH–cofactor–substrate). Incomplete conformational holo transition could explain the low catalytic efficiency (k_{cat}/K_M) and higher K_D values of mutants toward NAD and NADP since only the full inherent binding energy is available when the transition is complete. The best value for k_{cat}/K_M and K_D for NADP was observed for the D32A–S mutant. On this triple mutation there is a switch in favor of NADP specificity, but with a k_{cat}/K_M ratio 50-fold less than that observed for the wild type with NAD.

Comparison of the K_2/K_1 ratios for NAD binding to D32X mutants (Asn, Glu) suggests that position 32 could play an essential role for cooperative behavior. Replacing Asp32 by Glu reveals positive cooperativity for the first two subunits. This was not expected and remains to be explained at the molecular level. The results support the hypothesis of a predominant role of the adenosine subsite in revealing cooperative properties provided a right positioning of the nicotinamide moiety of NAD as already suggested (Schlessinger & Levitski, 1974; Schlessinger et al., 1975; Henis & Levitski, 1977, 1980; Glöggler et al., 1982). All the mutants seem to bind the pyridinium ring in a fashion similar to that of the NAD in the wild type. There are three arguments in favor

of this hypothesis. First, the total fluorescence quenching of GAPDH in the presence of NAD(P) versus apoGAPDH is similar for all mutants, suggesting a similar positioning of the pyridinium ring toward Trp310 in all mutants. Second, the positioning of the nicotinamidium moiety of NADP bound to the S mutant superimposes well with that of the NAD in the wild-type structure (Wonacott, unpublished results). Third, the inactivation rate of GAPDH by 3-CAPAD is similar for the wild type, D32A, S, and D32A–S mutants, thus indicating a similar orientation of the alkylating function situated at the C3 position of the pyridinium toward Cys149. However, cooperative properties are depending on the nature of the mutations. This strongly suggests that (1) a partial efficient binding of the adenosine ring does not prevent a correct positioning of the pyridinium ring and (2) a correct positioning of the pyridinium ring is necessary but not sufficient for inducing cooperativity.

As mentioned above, other mutagenesis studies have probed NAD(P) specificity (Chen et al., 1991; Fan et al., 1991; Feeney et al., 1990; Scrutton et al., 1990; Nishiyama et al., 1993; Bocanegra et al., 1993). Clearly, all the reports support the idea that many residues contribute to binding and specificity for the NAD(P) coenzyme and that the same mutation introduced in the adenosine subsite of different dehydrogenases can give quite different effects on the binding and the catalytic constants. Therefore, it is difficult from a rationale to predict all the structural factors that could be implicated in determining specificity for a coenzyme. This is particularly the case for GAPDH where specificity also depends on structural determinants situated at subunit interfaces of the tetramer. Only structural data on the mutants with both cofactors bound can reveal the precise role of the amino acid residues which influence coenzyme specificity.

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