Phosphorus-31 Nuclear Magnetic Resonance Studies on Coenzyme Binding and Specificity in Glyceraldehyde-3-Phosphate Dehydrogenase†

J. Eyschen,‡ B. Vitoux,‡ S. Rahuel-Clermont,‡ M. Marraud,§ G. Branlant,‡ and M. T. Cung*,§

Laboratoire d'Enzymologie et de Génie Génétique, Université Henri Poincaré Nancy I, URA CNRS 457, BP 239, 54506 Vandoeuvre-lès-Nancy Cedex, France, and Laboratoire de Chimie Physique Macromoléculaire, URA CNRS 494, ENSIC-INPL, 1, rue Grandville, BP 451, 54001 Nancy Cedex, France

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ABSTRACT: Binding of NAD(P)$^+$ to wild type and a series of mutants of the glycolytic NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Bacillus stearothermophilus* designed to alter the cofactor specificity [Clermont, S., Corbier, C., Mely, Y., Gerard, D., Wonacott, A., & Branlant, G. (1993) *Biochemistry 21*, 10178−10184] has been studied by ^{31}P NMR. In the mutants with the L187A and P188S substitutions, the pyrophosphate signals are split, and the upfield resonance has been assigned to the P(a) phosphate. Titration of the NADP$^+$ 2′-phosphate pK_a deduced from its chemical shift shows that the electrostatic environment in the binding site is largely affected by the single point mutations. pK_as ranging from 7.7 for the L187A-P188S mutant to <5.7 for the D32G-L187A-P188S and D32A-L187A-P188S mutants have been observed, thus indicating that the binding of NADP$^+$ is modulated by the ionization state of its 2′-phosphate. In the quintuple mutant L33T-T34G-D35G-L187A-P188S, designed in comparison with the photosynthetic NAD(P)-dependent GAPDH of the chloroplast, the 2′-phosphate has a pK_a of 6.8. As further stabilizing interactions like hydrogen bonds or positively charged side chains would lower this pK_a, it is suggested that the 2′-phosphate ionization state of bound NADP$^+$ in chloroplastic GAPDH is dianionic. The NADP$^+$ dissociation rate constants (k_{off}) of the three mutants D32G, L187A-P188S, and D32G-L187A-P188S are higher at pH 6.1 than at pH 8.1 and are similar at the same pH, indicating that the difference in binding affinity between these three mutants results from the molecular recognition step or a conformational change upon binding.

Nicotinamide adenine dinucleotide (NAD)[1] and its analogue NADP, esterified with phosphate at the 2′-hydroxyl group of the adenosine moiety, are two ubiquitous cofactors widely distributed in living organisms where they mainly participate in oxidoreductive processes (Stryer, 1988).

X-ray crystallographic studies carried out on NAD(P)-dependent oxidoreductases have revealed the occurrence of structural domains responsible for cofactor binding and orientation, a prerequisite for an efficient hydride transfer during catalysis (Rossmann et al., 1975; Eklund & Brändén, 1987). The full characterization of a dinucleotide-binding motif in lactate dehydrogenase was first proposed in the early seventies (Adams et al., 1970a,b). Since that time, this domain, traditionally known as the "Rossmann fold", has been observed in numerous dehydrogenases and reductases specific for either NAD or NADP (Mathews, 1991; Schulz, 1992). The canonical Rossmann fold is composed of a central six-stranded parallel β-sheet flanked on each side by two α-helices, thus corresponding to a $\beta\alpha\beta\alpha\beta$ topology, repeated twice at the primary structure level. When bound, NAD(P) is located at the β-sheet edge which is defined by the loops separating each strand from the next helix along the polypeptide chain (Eklund & Brändén, 1987).

A systematic overview of NAD(P)-dependent oxidoreductases has revealed that most of these enzymes exhibit a strong preference for either NAD or NADP, in agreement with the distinct metabolic functions generally assigned to NAD (catabolic role) and NADP (anabolic role) (Stryer, 1988). Given that the polypeptide main chains in all Rossmann folds are nicely superimposable (Eklund & Brändén, 1987), the discrimination between NAD and NADP is likely due to molecular determinants belonging to the side chains which define the inner surface of the cofactor binding cleft.

A consistent explanation of the cofactor specificity has not yet emerged because of the various local particularities in the coenzyme binding cleft of each enzyme. Furthermore, putative long-range effects due to distant side chains cannot be easily appreciated through sequence alignments or even visual inspection of the three-dimensional structures. The influence of such long-range effects on substrate specificity has been demonstrated in the case of serine proteases (Hedstrom et al., 1992, 1994): changing trypsin into a chymotrypsin-like protease requires mutation of some of the residues of the substrate binding pocket in conjunction with other substitutions in two surface loops. In this context, the NAD(P)-dependent oxidoreductases which exhibit similar

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* To whom correspondance should be addressed.

‡ Laboratoire d'Enzymologie et de Génie Génétique.

§ Laboratoire de Chimie Physique Macromoléculaire.

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[1] Abbreviations: GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; NAD(P), nicotinamide adenine dinucleotide (phosphate) oxidized or reduced form; NAD(P)$^+$, nicotinamide adenine dinucleotide (phosphate) oxidized form; EDTA, ethylenediaminetetraacetic acid; wt, wild type; D32G, Asp32→Gly; D32A, Asp32→Ala; S, Leu187→Ala, Pro188→Ser; B, Leu33→Thr, Thr34→Gly, Asp35→Gly.

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affinity for both NAD and NADP coenzymes constitute an interesting case, as their behavior cannot be simply explained in terms of electrostatic complementarity or repulsion between the protein and its cofactor but require consideration of other phenomena at the molecular recognition level. In this regard, recent investigations have been carried out on glutamate dehydrogenase (Baker et al., 1992a,b), glucose dehydrogenase (John et al., 1994), glucose-6-phosphate dehydrogenase (Rowland et al., 1994), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Corbier et al., 1990; Clermont et al., 1993).

The cofactor selectivity of GAPDH depends on its subcellular localization (Harris & Waters, 1976). The cytoplasmic GAPDH enzyme (EC 1.2.1.12) is involved in the glycolytic pathway and uses either specifically NAD (eucaryotes and eubacteria) or both cofactors NAD and NADP with a preference for NADP (archaebacteria). In addition, the chloroplastic GAPDH (EC 1.2.1.13) which contributes to photosynthetic CO_2 assimilation by the Calvin cycle exhibits a weak selectivity for NADP (Cerff, 1978; Ferri et al., 1978). The crystal structures of glycolytic GAPDH from various origins have been solved (Moras et al., 1975; Skarzynski et al., 1987; Korndörfer et al., 1995; Duée et al., 1996) and show essentially identical three-dimensional features at the secondary, tertiary, and quaternary structural levels. Although the crystal structure of a chloroplastic GAPDH has not been determined yet, a sequence comparison between chloroplastic enzymes and glycolytic enzymes of eubacteria reveals a high degree of homology (Martin et al., 1993) and strongly suggests that both glycolytic and chloroplastic GAPDHs adopt similar native folding. Thus, the high resolution crystal structure of the glycolytic GAPDH of the moderate thermophile *Bacillus stearothermophilus* (Skarzynski et al., 1987) can be reliably used as a starting model to switch the cofactor selectivity from NAD to NADP by modifying the amino acid positions supposed to be critical to this purpose.

In two preceding papers (Corbier et al., 1990; Clermont et al., 1993) we have designed and characterized a series of mutants of *B. stearothermophilus* GAPDH obtained by single or multiple point mutations in two regions surrounding the adenosine 2′ position. One of these regions corresponds to the loop following the second β strand of the first $\beta\alpha\beta\alpha\beta$ motif (see above), whereas the other is included in the so-called "S-loop" fragment, a specific structural feature of GAPDH close to bound NAD(P), which contributes to the quaternary interactions found in this homotetrameric enzyme (Skarzynski et al., 1987).

These results, in conjunction with those obtained more recently on two other mutants (Clermont, 1994), have demonstrated that partial reversal of the cofactor selectivity can be rationally achieved, but often at the cost of a severe decrease in catalytic efficiency when compared with wild-type enzymes. This observation holds also true for other oxidoreductases for which the cofactor selectivity has been reversed: NADP-dependent glutathione reductase (Scrutton et al., 1990; Perham et al., 1991; Bocanegra et al., 1993), NAD-dependent malate dehydrogenase (Nishiyama et al., 1993), NAD-dependent yeast alcohol dehydrogenase (Fan et al., 1991), and NAD-dependent isopropylmalate dehydrogenase (Miyazaki & Oshima, 1994).

Thus, in order to explore the environment of the cofactor phosphate groups and gain some information about the binding affinity of NAD^+ or $NADP^+$ bound to these mutants, we have undertaken a series of ^{31}P nuclear magnetic resonance (NMR) studies. ^{31}P NMR spectroscopy is a well-suited method for the analysis of small phosphorus-containing ligands bound to a protein (Gorenstein, 1984), because it gives access to various parameters such as (i) the pK_a of discrete phosphate groups, (ii) the dissociation constants K_D in case of a slow exchange between the free and bound forms of the ligand, and (iii) the kinetic constants k_{off} in suitable conditions.

In this work, we have deduced the exchange condition and, for three mutants, the dissociation rate constant for NAD(P) between its free and enzyme-bound state from the variation of the resonance shape and line width with increasing cofactor concentration. In conjunction with the 2′-phosphate pK_a determined via the chemical shift of the bound resonance, we draw some conclusions about the nature of the 2′-phosphate binding site and the reasons for the low cofactor affinity in the mutant enzymes.

MATERIALS AND METHODS

Isolation of Wild-Type and Mutated Enzymes. Production and purification of wild-type and mutant *B. stearothermophilus* enzymes were performed as described earlier (Corbier et al., 1994). The apoenzyme was prepared by activated charcoal treatment (Racker & Krimsky, 1952). Protein concentrations were determined spectrophotometrically, using molar extinction coefficients of 1.17×10^5 and 1.31×10^5 M^{-1} cm^{-1} for the apo- and holoenzyme, respectively. Enzyme activity was checked during purification, before and after each experiment, by measuring the initial rate of appearance of NAD(P)H at 340 nm on a Kontron Uvikon spectrophotometer (Ferdinand, 1964).

Sample Preparation. The apoenzyme was dialyzed against a constant ionic strength buffer of 0.1 M (33 mM succinate, 44 mM imidazole, 44 mM diethanolamine, 5 mM EDTA) at a pH adjusted to the desired value by addition of KOH or HCl and concentrated on an Amicon YM30 membrane. NAD or NADP were then added to reach a final concentration of 0.33−10 mM for a GAPDH concentration of 0.5 mM (subunit concentration). The sample was dissolved in 2 mL of water containing 10% D_2O for signal lock.

^{31}P NMR. The NMR spectral data were obtained on a Bruker AC200 spectrometer operating at 81 MHz. All experiments were carried out at 25 °C under broad-band decoupling conditions with a spectral width of 5000 Hz, 4K data points, a 45° flip angle, and a 0.41 s acquisition time. Typical data acquisition involved the accumulation of 1000−16000 transients, requiring 10 min to 3 h of instrument time. Chemical shifts were referenced to the external 85% phosphoric acid. The pH was readjusted after each addition of cofactor, and its value was not corrected for the deuterium isotope effect.

Data Processing and Analysis. A 10 Hz line broadening exponential multiplication was applied to the free induction decay prior to Fourier transformation. The chemical shifts and line widths of the 2′-phosphate resonance for the free and bound NADP were obtained after deconvolution with the Bruker WINNMR software. Titration curves for these resonances were analyzed by using a nonlinear least-squares curve fitting procedure based on Marquardt's algorithm (Marquardt, 1963) assuming a simple proton dissociation

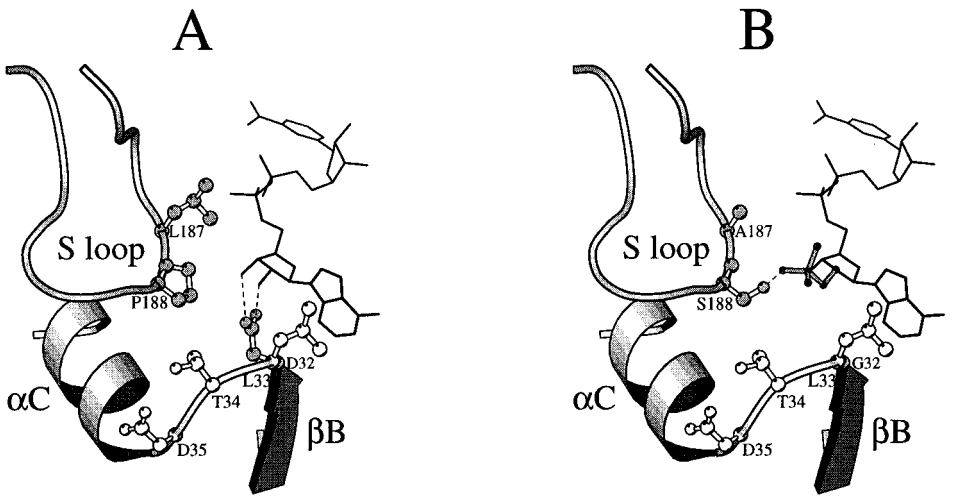

FIGURE 1: Representation of the adenosine ribose subsite of *B. stearothermophilus* GAPDH generated from the PDB 1gd1 entry. (A) wild-type enzyme with NAD^+, (B) simulation of the D32G-S mutant with $NADP^+$. $NAD(P)^+$ and the residues from βB to αC belong to the O subunit while the S loop is part of the R subunit. The figure was generated with RasMol (R. Sayle, RasMol: A program for the visualisation of protein and nucleic acid structures, ftp://ftp.dcs.ed.ac.uk/pub/rasmol) and MOLSCRIPT (Kraulis, 1991).

model:

$$\text{NAD-PO}_4^{2-} + \text{H}^+ \rightleftarrows \text{NAD-PO}_4\text{H}^- \qquad (1)$$

$$\delta_{obs} = (\delta_m + \delta_d 10^{(pH-pKa)})/(1 + 10^{(pH-pKa)}) \qquad (2)$$

where δ_m and δ_d are the chemical shifts of the 2′-phosphate in the monoanionic and dianionic form, respectively.

Exchange Theory. We are concerned with an equilibrium in which the NAD or NADP cofactor exchanges between a free and enzyme-bound state. The exchange rate influences directly on the shape of the ligand nuclei resonances (Pople et al., 1959). If we consider a ligand L, bound to an enzyme E, and forming the complex EL:

$$\text{E} + \text{L} \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} \text{EL} \qquad (3)$$

the dissociation constant K_D is defined by

$$K_D = [\text{E}][\text{L}]/[\text{EL}] = k_{off}/k_{on} \qquad (4)$$

and the lifetimes of the free (τ_F) and bound ligand (τ_B) are given by

$$1/\tau_F = k_{on}[\text{E}] \qquad 1/\tau_B = k_{off} \qquad (5)$$

Different exchange conditions should be considered: If the exchange of a given nucleus between the two states is slow, i.e., $2\pi|\delta_F - \delta_B| \gg (1/\tau_F + 1/\tau_B)$ (where δ_F and δ_B are the chemical shifts of the free and bound ligand), a resonance is observed for each of the free and bound ligands. The line widths of the free ($\Delta\nu_{1/2F}$) and bound ($\Delta\nu_{1/2B}$) ligand resonances are then given by

$$\pi\Delta\nu_{1/2F} = 1/T_{2F} + 1/\tau_F = 1/T_{2F} + P_B k_{off}/P_F \qquad (6)$$

$$\pi\Delta\nu_{1/2B} = 1/T_{2B} + k_{off} \qquad (7)$$

where $1/T_{2F}$ and $1/T_{2B}$ are the spin-spin relaxation rates, and P_F and P_B the ligand molar fractions of the free and bound states according to

$$P_B = [\text{EL}]/([\text{EL}] + [\text{L}]) = 1 - P_F \qquad (8)$$

Table 1: Nature of the Mutants Studied and Exchange Condition Observed for $NADP^+$ between Its Free and Enzyme-Bound Form

	type of residue in position[a]						
enzyme	32	33	34	35	187	188	exchange condition
wild-type	D	L	T	D	L	P	fast
D32G mutant	G						slow
S mutant					A	S	slow
D32A-S mutant	A				A	S	intermediate
D32G-S mutant	G				A	S	slow
B-S mutant		T	G	G	A	S	slow

[a] Residues 32−35 form a loop between β-strand B and α-helix C; residues 187 and 188 belong to the S-loop. In the mutants only the positions that have been changed with respect to the wild-type sequence are indicated.

From these equations, the line width for the bound ligand is independent of the ligand concentration, while the line width for the free ligand decreases with increasing ligand concentration. $\Delta\nu_{1/2F}$ versus P_B/P_F gives a linear plot with a slope of $(1/\pi)k_{off}$.

If the exchange is fast, i.e. $2\pi|\delta_F - \delta_B| \ll (1/\tau_F + 1/\tau_B)$, a single ligand resonance is observed at a "weighted average" chemical shift δ_{obs} given by

$$\delta_{obs} = P_F\delta_F + P_B\delta_B \qquad (9)$$

For an intermediate exchange rate, i.e., $2\pi|\delta_F - \delta_B| \approx (1/\tau_F + 1/\tau_B)$, the line shape becomes complex, and there is no simple relation giving the frequency for which the line width amplitude goes through a maximum. In order to distinguish between fast and intermediate exchange, we have plotted the observed line widths as a function of the ligand concentration. According to Feeney et al. (1979), when the line width shows a monotonic decrease rather than a maximum with increasing ligand concentration, the exchange contribution is small, thus corresponding to a fast exchange [for a detailed discussion, see Feeney et al. (1979)].

RESULTS

Nature of the Mutations. The positions mutated in the wild-type enzyme and the nature of the residues chosen for the substitutions are indicated in Figure 1 and Table 1. The mutant enzymes (Clermont, 1994) have been designed by

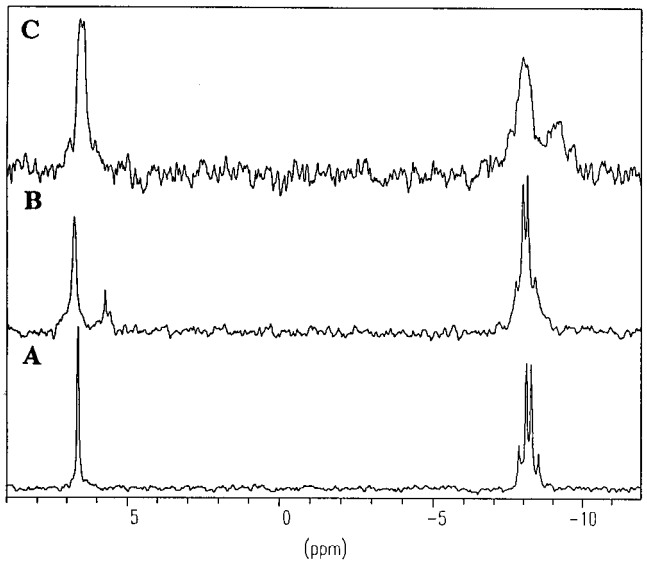

FIGURE 2: 81 MHz ^{31}P NMR spectra of NADP$^+$ (A) free in solution, (B) in the presence of wild-type GAPDH, and (C) in the presence of the D32G-S mutant. The spectra in the presence of an enzyme were obtained with a NADP$^+$/enzyme subunit ratio of 2:1. [The small peak at 5.6 ppm in the central spectrum (B) is not observed in other spectra with wild-type enzyme and corresponds to a contamination].

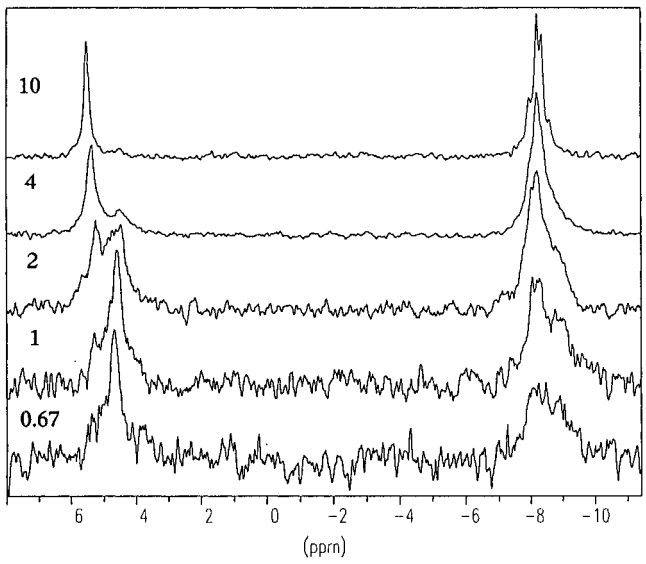

FIGURE 3: ^{31}P NMR spectra of NADP$^+$ in the presence of the quintuple mutant B-S as a function of NADP$^+$ concentration. The ratio of NADP$^+$ to enzyme is expressed as moles of NADP$^+$ per mole of enzyme subunits.

taking into account the fingerprint described for NAD(P) binding oxidoreductases (Wierenga et al., 1985) (presence of a negatively charged residue in the loop after the second β strand for NAD-dependent enzymes, absence of this residue in NADP-dependent enzymes; position 32 in GAPDH) on the one hand and the amino acid sequence found in chloroplastic GAPDH (positions 33, 34, and 35 in the loop after the second β strand and positions 187 and 188 in the "S-loop") on the other hand.

^{31}P NMR Spectra of Free and Enzyme-Bound NADP$^+$. The ^{31}P NMR spectrum of NADP$^+$ free or bound to the wild-type GAPDH is shown in Figure 2. The signal at 6.6 ppm corresponds to the 2′-phosphate while the pyrophosphate resonances appear at -8.0 and −8.4 ppm (Feeney et al., 1975). A $^2J_{PP}$ coupling constant of 20.1 Hz is observed for the free NADP$^+$. Upon binding to the wild-type enzyme, the ^{31}P chemical shift and the $^2J_{PP}$ coupling constant are not significantly modified, except a substantial peak broadening due to NADP$^+$ complexation by the macromolecule. In contrast, upon binding to the D32G-S mutant, one pyrophosphate resonance is upfield shifted by 1 ppm. Such an upfield shift is only observed with the GAPDH mutants with S substitution and not for any others. In order to determine whether this shift is specific for NADP$^+$, we have run the ^{31}P spectrum of NAD$^+$ in the presence of the wild-type and S mutant enzymes. In this cofactor, the pyrophosphate resonances are also superimposed at −8.5 ppm in the former case and split at −8.2 and −9.0 ppm in the latter case.

Determination of the Exchange Conditions. The NADP$^+$ exchange rate in the presence of wild-type GAPDH and five mutant enzymes was estimated by varying the NADP$^+$/enzyme subunit ratio from 0.67 to 10 and by following the position and line shape of the 2′-phosphate resonance (Table 1). As the exchange rate may depend on the pH, all experiments were repeated at pH 8 and 6. For the wild-type enzyme at pH 8, the single resonance for the 2′-phosphate does not shift, but its line width decreases monotonically when NADP$^+$ concentration is raised. This supports that NADP$^+$ is in fast exchange, while NADP$^+$ in the free or in the bound state has a quite similar chemical shift. Interestingly, at pH 6, the 2′-phosphate signal is so broad that it does not emerge from the noise for low cofactor/enzyme ratios (0.67 and 1). This seems to indicate that the exchange rate becomes slower at lower pH.

Figure 3 shows the spectrum of NADP$^+$ in the presence of the quintuple mutant B-S. The two distinct resonances for the 2′-phosphate, which do not shift with increasing cofactor concentration, clearly indicate that exchange of NADP$^+$ between the free and bound states is slow. At pH 6.2, the upfield signal is not affected by increasing NADP$^+$ concentration while the downfield signal not only increases in intensity but also becomes sharper. Therefore, the upfield signal can be assigned to bound NADP$^+$ and the downfield signal to free NADP$^+$. At pH 7.8, the same comment applies, except that the upfield signal corresponds to free NADP$^+$ and the downfield signal to bound NADP$^+$. Similar spectra are observed for the other mutant enzymes with one exception, the D32A-S mutant, where both distinct signals observed for the free and bound 2′-phosphate resonances at low cofactor content shift with increasing NADP$^+$ concentration, thus reflecting a case of intermediate exchange behavior (spectra not shown).

pH Titration of the 2′-Phosphate Resonance of NADP$^+$in the Presence of Wild-Type and Mutant GAPDHs. ^{31}P chemical shifts are very sensitive to the phosphate protonation state. In order to characterize the environment of the 2′-phosphate group and gain some information about its binding mode and ionization state, we have run the spectrum of NADP$^+$ in the presence of wild-type and mutant enzyme over a pH range of 5.8−8.5 with a NADP$^+$/enzyme subunit ratio of 2. All spectra obtained are characteristic of slow exchange conditions over the pH range studied, except in the case of the wild-type enzyme, for which the exchange is fast, and of the D32A-S mutant, for which the contribution of the free NADP$^+$ was minimized by working with a NADP$^+$/enzyme subunit ratio equal to 1. As an example, Figure 4 shows the evolution of the chemical shifts of the

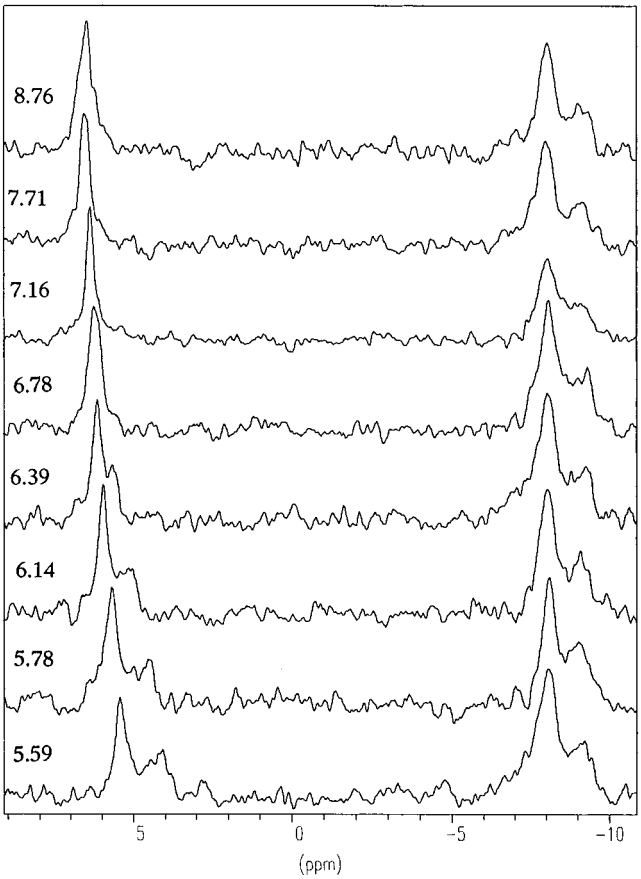

FIGURE 4: ^{31}P NMR spectra of NADP$^+$ in the presence of the D32G-S mutant as a function of the pH. The NADP$^+$/enzyme subunit ratio is 2:1.

Table 2: Variables Obtained after Curve Fitting of the ^{31}P NMR Results

	2′-phosphate of free NADP$^+$			2′-phosphate of bound NADP$^+$		
experience	pK_a	δ_m (ppm)[a]	δ_d (ppm)[a]	pK_a	δ_m (ppm)[a]	δ_d (ppm)[a]
NADP$^+$	5.9	2.92	6.65	/	/	/
+ wild-type enzyme	/	/	/	6.5	nr[b]	7.07
+ D32G mutant	6.1	nr[b]	6.73	6.4	nr[b]	7.38
+ S mutant	6.3	nr[b]	6.72	7.7	3.27	6.96
+ D32A-S mutant	/	/	/	<5.7	nr[b]	6.63
+ D32G-S mutant	6.0	nr[b]	6.62	<5.7	nr[b]	6.45
+ B-S mutant	6.2	nr[b]	6.67	6.8	3.59	7.24

[a] δ_m and δ_d are the chemical shifts of the 2′-phosphate in the monoanionic and dianionic ionization state, respectively. [b] nr, values are not reported because of their low accuracy.

free and bound 2′-phosphate resonances as a function of the pH for the D32G-S mutant. In Table 2 the pK_a values are listed along with the upper and lower chemical shift values obtained after data computer fitting.

The pK_a value obtained for pure NADP$^+$ (pK_a = 5.9) is slightly lower than the average value obtained for free NADP$^+$ in the presence of an enzyme (pK_a = 6.15 ± 0.15). For the enzyme-bound NADP$^+$, the mutations strongly influence the pK_a of the 2′-phosphate. There is a difference of more than two pH units between the S mutant and the two triple mutants D32G-S and D32A-S.

When NADP$^+$ binds to the wild-type enzyme or the D32G mutant, the pK_a of the 2′-phosphate group does not change noticeably with respect to free NADP$^+$, thus suggesting a similar environment of the 2′-phosphate group in both enzymes. In contrast, the 2′-phosphate pK_a for the S mutant increases noticeably, leading to a preference for the monoanionic state at the physiological pH, while the intensity of the bound signal decreases when increasing the pH. When D32 is also mutated (D32G-S and D32A-S), the pK_a drops to a value of less than 5.7, denoting a large influence on the 2′-phosphate pK_a of the D32 substitutions. In the B-S mutant, the pK_a slightly increases in comparison with the wild-type enzyme, but the dianionic form remains the preferred ionization state at the physiological pH.

Table 3: Dissociation Rate Constants of NADP$^+$ Determined by ^{31}P NMR

	k_{off} (s^{-1})[a]		% of dianionic 2′-phosphate[b]	
enzyme	pH 6.1	pH 8.1	pH 6.1	pH 8.1
D32G	152	77	33.4	98.4
S	392	111	2.5	66.6
D32G-S	106	68	76.0	100

[a] k_{off} values are deduced from the experimental line width of the free NADP$^+$ 2′-phosphate resonance. [b] Calculated from pH = pK_a + log(d/m), where d and m are the proportions of the dianionic and monoanionic form, respectively.

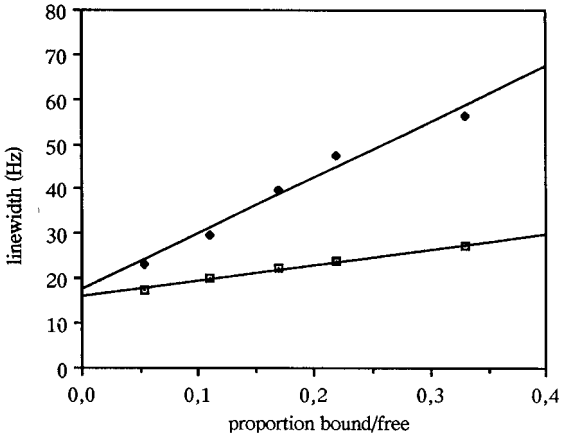

FIGURE 5: Line width of the 2′-phosphate resonance of free NADP$^+$ in the presence of the S mutant as a function of P_B/P_F according to eq 6. (□) pH 8.0, (◆) pH 6.1.

The chemical shift of the dianionic 2′-phosphate varies for the different enzymes in a range of about 1 ppm around the free NADP$^+$ value. For the two enzymes for which the chemical shift of the monoanionic phosphate can be determined with enough accuracy (S and B-S), the $\delta_d - \delta_m$ shift denoting phosphate protonation is of the same order as for the free NADP$^+$.

NADP$^+$/Enzyme Interaction. The dissociation rate constants (k_{off}) of the binary complex NADP$^+$/GAPDH have been determined from the NADP$^+$ 2′-phosphate ^{31}P resonance line width for the three mutants D32G, S, and D32G-S, at two pH values (6.1 and 8.1), in order to investigate the influence of the ionization state of the 2′-phosphate on the NADP$^+$ kinetic behavior (Table 3). A series of spectra, with cofactor/enzyme subunit ratios of 3−20, have been recorded, and the line width of the free NADP$^+$ 2′-phosphate ^{31}P resonance has been evaluated by fitting a Lorentzian curve to the experimental peak. Figure 5 shows the line width plotted versus P_B/P_F for the S mutant according to eq 6. A straight line is obtained for both pH values, and the k_{off} values estimated from the slope are indicated in Table 3. For the

Table 4: Kinetic Parameters, Dissociation Constants, and Molar Extinction Coefficients of NAD(P) for Wild-Type and Mutant GAPDHs

	K_M (mM)[a]		k_{cat} (s^{-1})[a]		K_D (μM)[b]		Racker band (M^{-1} cm^{-1})[c]	
enzyme	NAD^+	$NADP^+$	NAD^+	$NADP^+$	NAD^+	$NADP^+$	NAD^+	$NADP^+$
wild-type	0.1[d]	na[f]	70[d]	na[f]	0.9[d]	35[d]	1100[e]	0[e]
D32G mutant	0.4[e]	2.1[e]	6[e]	22[e]	61	6.7	280	550
S mutant	0.4[d]	7.1[d]	70[d]	15[d]	1.1[d]	36[d]	1100[e]	600[e]
D32A-S mutant	0.8[d]	1.3[d]	7[d]	18[d]	126[d]	3.4[d]	430[e]	1020[e]
D32G-S mutant	1.3[e]	0.9[e]	6[e]	19[e]	17	1.3	620[e]	1060[e]
B-S mutant	0.8[d]	1.3[d]	80[d]	18[d]	4.4	20.8	1100[e]	720[e]

[a] Kinetic parameters were determined at 22 °C in 40 mM triethanolamine, 50 mM phosphate, and 0.2 mM EDTA buffer, pH 8.9. [b] Dissociation constants were determined by affinity labeling with 3-(chloroacetyl)pyridine adenine dinucleotide at 22 °C in 0.1 M phosphate buffer, pH 8.5 (Corbier et al., 1990). [c] Molar extinction coefficients of the Racker band were determined in 0.1 M Tris and 0.02 M EDTA buffer, pH 8. The Racker band corresponds to an absorbance at 360 nm due to a charge transfer complex between the cofactor nicotinamidium ring and the residue Cys 149 (Racker & Krimsky, 1952). [d] Clermont et al. (1993). [e] Clermont (1994). [f] Not active.

three enzymes, k_{off} increases from the low to high pH. Both D32G and D32G-S mutants give rather similar k_{off} values, inferior to that for the S-mutant which also experiences the maximum increase, by a factor of 3.5, with pH decrease.

DISCUSSION

Assignment of the Pyrophosphate Signals. Upon binding to the S, D32G-S, D32A-S, and B-S GAPDH mutants, one of the two $NADP^+$ pyrophosphate signals experiences an upfield shift of about 1 ppm, thus giving two separate resonances. A larger magnetic nonequivalence is even observed for $NADP^+$ bound to isocitrate dehydrogenase (Mas & Colman, 1984), dihydrofolate reductase (Hyde et al., 1980), NADPH-cytochrome P-450 reductase (Otvos et al., 1986), and NAD^+ bound to UDP-galactose 4-epimerase (Konopka et al, 1989).

^{31}P chemical shifts are sensitive to the intrinsic conformational properties of the molecule and, to a lesser degree, to the outer molecular environment (Gorenstein, 1984). Concerning conformation, Gorenstein has proposed an empirical correlation between ^{31}P chemical shifts and the O−P−O angles for phosphate esters (Gorenstein, 1975) and further suggested that the ^{31}P chemical shifts are sensitive to the torsional angles ω and ω' which describe the rotation around each pyrophosphate P−O bond relative to the O−P−O plane (Gorenstein & Kar, 1975). Concerning molecular environment, Lerner and Kearns (1980) have reported detailed studies of the solvent effects on several cyclic nucleotides having a more or less rigid conformation of the phosphodiester group. They found that the upfield shift is inversely proportional to the number of hydrogen bonds between the phosphate and the solvent. This correlation has been used for $NADP^+$ bound to dihydrofolate reductase (Gerothanassis et al., 1991) to predict that the upfield signal is due to the 5′-phosphate linked to adenosine (P(a)). This attribution has been confirmed later by two-dimensional heteronuclear ^{1}H−^{31}P NMR spectroscopy (Gerothanassis et al., 1992).

Thus, the observed shift could denote some conformational modifications of $NADP^+$ bound to the enzyme with the S mutation compared with NAD^+ bound to wild-type enzyme (a reduction of the O−P−O angle or a 50° rotation of one of the P−O torsional angles) or a change in cofactor environment (or a combination of both effects). The crystallographic structure of the S mutant with $NADP^+$ (resolution 2.7 Å, final *R*-factor 16%) shows no significant change in the pyrophosphate conformation relative to the wild-type enzyme with NAD^+ (Wonacott, personal communication). On the other hand, the L187 and P188 residues are close to the pyrophosphate bridge of the bound cofactor, especially near the P(a) phosphate, and their mutation could result in a modified pyrophosphate environment. In particular, the number of hydrogen bonds involving the P(a) phosphate is considerably reduced in the S mutant: because of a small displacement of N180, the hydrogen bond with O1P(a) is disrupted and the O2P(a) is no more hydrogen-bonded to water, so that only two bonds out of six for wild-type GAPDH remain. The bonding pattern of the P(n) phosphate is only slightly affected by the mutations: one hydrogen bond with a water molecule is disrupted. Therefore, it seems to us reasonable to tentatively assign the most shifted signal, i.e., the upfield resonance, to the P(a) phosphate in the mutant enzymes.

Ionization State of Bound NADP$^+$. The pH dependence of the 2′-phosphate ^{31}P resonance informs us on the protein site closely surrounding the extra phosphate and the local interactions between the two partners for the different mutant enzymes (Gorenstein, 1984).

For $NADP^+$ bound to the wild-type enzyme, the 2′-phosphate pK_a is quite near the value for the free form. In conjunction with the high dissociation constant for $NADP^+$ and the absence of a Racker band (Table 4), such a result suggests that the wild-type enzyme has no specific binding site for the extra phosphate and probably maintains it largely exposed to the surrounding solvent because of an electrostatic repulsion with the D32 residue and steric hindrances with the side chains in positions 187 and 188.

Mutation of D32 to G apparently has no effect on the pK_a of this group with respect to the wild-type data. This could mean that the 2′-phosphate largely remains accessible to the surrounding solvent as in wild-type GAPDH. However, the smaller K_D value is in agreement with the release of the electronic repulsion due to D32 in wild-type GAPDH.

According to molecular modeling performed on the wild-type enzyme, some of the steric constraints between the 2′-phosphate and the enzyme are released in the S mutant (substitution of L187 and P188 by Ala and Ser, respectively). The bound $NADP^+$ pK_a increases to 7.7 and indicates that the 2′-phosphate lies in the vicinity of negatively charged groups which could be the D32, and perhaps D35, β-carboxylate. The pK_a increase and the intensity decrease of the bound signal with increasing pH denotes that the 2′-phosphate monoanionic form is more stable at neutral pH than the dianionic one.

Substitution of D32 by A or G in the two triple mutants D32G-S and D32A-S reduces the 2′-phosphate pK_a to less

than 5.7. The phosphate dianionic state is then much more stable than the monoanionic form at neutral pH. Although there seems to be no positively charged residue close enough to interact directly with the 2′-phosphate, such a behavior indicates that the overall electrostatic environment in the extra phosphate site is positive. The three-dimensional structure of GAPDH (Skarzynski et al., 1987) reveals the existence of five arginine residues (R10, R13, R77, R98, and R183) situated at less than 15 Å from the 2′-oxygen position, thus possibly contributing to the positive local electrostatic field. In the dihydrofolate reductase (Hyde et al., 1980) and isocitrate dehydrogenase (Mas & Colman, 1984), titration of the 2′-phosphate by ^{31}P NMR between pH 5.5 and 8 has shown no shift of the bound signal which corresponds to the dianionic phosphate. Hence, the dianionic ionization state of $NADP^+$ bound to these enzymes is more stable than in our triple mutants suggesting that binding of $NADP^+$ to GAPDH could be improved by introduction of a positive charge in the adenosine subsite.

It is interesting to note that the acidic residue found in NAD-dependent oxidoreductases (where it interacts with the free 2′- and 3′-hydroxyl groups of the adenosine ribose) is conserved in the NAD(P)-dependent GAPDH and glutamate dehydrogenases. Two explanations have been put forward up to now (Baker et al., 1992b): on one side, the acidic residue could be involved in a water-mediated hydrogen bond with the 2′-phosphate or, on the other side, it could increase the 2′-phosphate pK_a in such a manner that the phosphate would become protonated in the site and then would form a direct hydrogen bond with it. In *B. stearothermophilus* GAPDH, the first, second, and third residues after D32 are L33, T34, and D35. They are replaced by a Thr (or Ser) and two Gly residues in chloroplastic GAPDH, thus creating, if there is no conformational difference between both enzymes, a cavity adjacent to D32 that could accept one or more water molecules and would allow a hydrogen bond between T33 (S33) and the phosphate (Corbier et al., 1990). The S mutant shows that the 2′-phosphate is able to change its preferred ionization state at neutral pH, when steric constraints due to the S loop are released, thus enabling a substantial proximity toward D32. However, the crystal structure of this mutant complexed with $NADP^+$ gives no evidence at all for a direct hydrogen-bonding interaction between the D32 β-carboxylate and the monoprotonated 2′-phosphate.

The B-S mutant has been designed on the basis of the NAD(P)-dependent chloroplastic GAPDH. The five residues that have been replaced in this mutant by their counterparts in the chloroplastic enzyme could form the binding site for the 2′-phosphate in the presence of D32. The surprisingly low Racker band value for $NADP^+$ in this mutant indicates that other residues are also involved in $NADP^+$ binding, either because they induce local rearrangements of the site or promote unexpected networks of noncovalent interactions. In comparison with the S mutant, further mutations in positions 33, 34, and 35 in the B-S mutant induce a 2′-phosphate pK_a drop from 7.7 to 6.8. Such a decrease can be hardly justified on the sole basis of the D35G mutation, because this residue probably lies 10 Å away from the 2′-phosphate position, whereas the closer D32 carboxylate remains present in this mutant. In fact, due to the removal of the side chains in positions 34 and 35 (mutations with Gly), a larger solvation of the phosphate group relative to the S mutant could furthermore contribute to this pK_a drop and thus support the first hypothesis of Baker (Baker et al., 1992b).

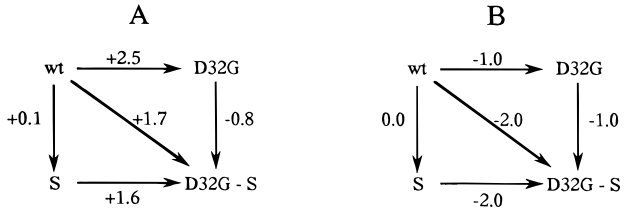

FIGURE 6: Thermodynamic box describing the free energy changes induced by the mutations. (A) GAPDH with NAD^+. (B) GAPDH with $NADP^+$. Free energy changes were calculated as followed: $\Delta\Delta G = -RT \ln(K/K')$ where K and K' are the dissociation constants before and after mutation, respectively.

Another role could be attributed to D32 in chloroplastic GAPDH: it could be involved in the orientation of a positively charged side chain toward the 2′-phosphate. In all presently known primary structures of chloroplastic GAPDH, there is in fact a strictly conserved Lys residue (K38) situated in the α-helix C after the loop containing D32. However, visual inspection of this region in the *B. stearothermophilus* enzyme shows that the equivalent residue in position 38 lies on the opposite side of the helix with respect to D32 location. This means that the conformation of the loop preceding helix C would have to be different in the chloroplastic GAPDH with respect to the glycolytic one in order to reorientate helix C. K38 could then interact simultaneously with the side chain of D32 and one of the $NADP^+$ 2′-phosphate oxygen atoms while in the apoenzyme it would hydrogen-bond exclusively to D32. If this hypothesis holds true, K38 would be responsible for a further decrease of the 2′-phosphate pK_a, so that the ionization state of bound $NADP^+$ would be dianionic in the chloroplastic enzyme.

Thermodynamical Aspects of the Mutations on the Enzyme Structure. Figure 6 shows the thermodynamic boxes describing the effect of mutating D32, L187, and P188 on the NAD^+ and $NADP^+$ binding energies (Fersht, 1985). For both cofactors bound to the enzyme, the effects of the mutations are nonadditive: substitution of one residue has not the same consequence when it takes place on the wild-type enzyme or the enzyme mutated on the other site. In the case of the NAD^+ bound enzyme, mutation of D32 generates a 2.5 kcal/mol energy loss, but the subsequent mutation of L187 and P188 is energetically favorable. In contrast, S substitution of the wild-type enzyme has a slight negative effect on the protein/cofactor affinity. In the presence of D32, such a behavior could be rationally explained by a gain of conformational flexibility for NAD^+ when substituting the bulky side chains of L187 and P188 by smaller ones. On the contrary, in the absence of D32, the loss of binding energy due to the absence of the two hydrogen bonds between D32 and the adenosine ribose could be partially compensated by the introduction of weaker hydrogen-bond contacts with the S188 hydroxyl group. In the case of $NADP^+$, mutation of L187 and P188 in the wild-type enzyme has no effect, and that of D32 has only a slight favorable positive effect on the overall binding energy whereas the additional mutation does increase it by 2 and 1 kcal/mol. Here again, one could imagine that S188 would be able to interact with $NADP^+$ in the absence of D32. In the S mutant crystallized with $NADP^+$, the repulsion between

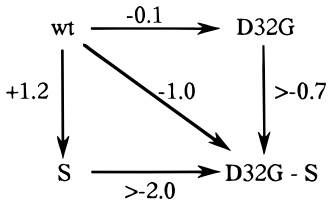

FIGURE 7: Box describing the effect of the mutations on the 2′-phosphate pK_a.

Table 5: Decomposition of the pK_a Variations Induced by the Different Mutations

mutation	D32 proximity due to steric constraint release	removal of distant D32	removal of close D32	hydrogen bond with S188	total
wt → D32G	/	−0.1	/	/	−0.1
wt → S	+1.2	/	/	/	+1.2
S → D32G-S	/	/	−1.2	>−0.7	>−2
D32G → D32G-S	/	/	/	>−0.7	>−0.7

D32 and the 2′-phosphate prevents the latter from coming into a favorable hydrogen-bonding distance with respect to S188. Once D32 removed, the conformation of the 2′-phosphate and maybe that of the adenosine ribose could change, thus allowing stabilization of the former.

The putative role of S188 is confirmed by the pK_a variations for the three mutants (Figure 7). The S mutations in the wild-type enzyme induce an increase of 1.2 unit in the 2′-phosphate pK_a whereas the same substitutions in the D32G mutant lead to a pK_a decrease. In the S mutant, as a result of releasing steric constraints, the 2′-phosphate comes close to D32, thus inducing a pK_a increase. In contrast, the decrease observed for the same substitution in the D32G mutant can only be explained either by a positively charged residue coming close to the 2′-phosphate, or by a stabilization by hydrogen bonding of the dianionic ionization state. As there is no basic residue close to the binding site, it seems more reasonable to incriminate a hydrogen bond between S188 and the 2′-phosphate that would be stronger with the di- than with the monoanion. The overall pK_a variations could then be attributed to the electrostatic effects due to the change in the environment when the 2′-phosphate goes from the solution to the binding site, taking into account the removal of D32, the release of steric hindrance with L187 and P188 and the formation of a hydrogen bond with S188 (Table 5).

Dissociation Kinetics of NADP$^+$. The dissociation rate constants (k_{off}) at two pH values show that, irrespective of the point mutations introduced in the D32G, S, and D32G-S mutants, NADP$^+$ dissociates more rapidly when its phosphate is monoanionic (Table 3). Whereas this result could be expected for the D32G and D32G-S mutants, it is rather surprising for the S mutant where the close D32 is supposed to favor the monoanionic over the dianionic phosphate. For that matter, the relative peak areas on the ^{31}P NMR spectra obviously demonstrate that the K_D is smaller at pH 6.1 than at pH 8.1. So the discrimination must occur during the cofactor/protein association step which is reflected by the association rate constants (k_{on}).

Similarly, the higher affinity of NADP$^+$ for the D32G-S mutant over the other ones is scarcely visible when looking at the k_{off} values. Thus, it is the difference between the respective association rate constants that should be at the origin of the difference between the overall affinities observed.

Thus, in all of the designed mutants of this study, it seems that the molecular recognition step of protein/cofactor association is the most affected by the different point mutations. In this context, it would be of interest to introduce a positively charged residue in the neighboring of the bound 2′-phosphate: this mutation should lead to a further decrease in the K_D value in relation with the modifications of the k_{on} (increase) and (or) k_{off} (decrease) values.

In conclusion, the different binding of NAD$^+$ and NADP$^+$ to the "Rossmann fold" appears to be the result of a very subtle equilibrium between repulsive and attractive phenomena, furthermore modulated in the case of NADP$^+$ by the ionization state of the 2′-phosphate. On the basis of the 3D structure of the NAD$^+$-bound wild-type GAPDH, we have rationally designed mutants where NADP$^+$ binding is substantially improved with respect to that measured with the glycolytic enzyme. In most of the mutants (S, D32G, D32G-S, D32A-S), the single or multiple point mutations in the wild-type enzyme have concerned positions contributing simultaneously to NAD$^+$ binding improvement (hydrogen bonding for D32 and van der Waals contacts for L187 and P188) and NADP$^+$-binding weakening (electrostatic repulsion for D32 and steric hindrance for L187 and P188). Thus, the mutations examined predominantly improve the molecular recognition step of the protein-NADP$^+$ complex (increase of k_{on} values) by removing some unfavorable interactions between the two partners. The side chain of S188 appears to be capable of introducing the only putative stabilizing contact with the 2′-phosphate, but this effect if any is only reflected by an almost negligible decrease of the k_{off} values (Table 3).

In the B-S mutant, chloroplastic residues 33, 34, and 35 supposed to act as positive determinants for the selective binding of NADP$^+$ versus NAD$^+$ proved to have practically no effect on NADP$^+$-recognition and binding (as shown by comparison of the Racker bands and K_D values for the S and B-S mutants). This means that a critical stabilizing interaction for NADP$^+$ in chloroplastic GAPDH is still lacking in this mutant: as a substantial binding-energy gain is expected to be only obtained via an attractive electrostatic interaction with the phosphate group, a properly oriented and positively charged residue is probably required to this purpose.

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