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Probing the Coenzyme Specificity of Glyceraldehyde-3-phosphate Dehydrogenases by Site-Directed Mutagenesis†

C. Corbier,‡ S. Clermont,‡ P. Billard,‡ T. Skarzynski,§ C. Branlant,‡ A. Wonacott,§ and G. Branlant*,‡
Laboratoire d'Enzymologie et de Gênie Gênêtique, URA CNRS 457, Faculté des Sciences, B.P. 239, 54506 Vandoeuvre les Nancy, France, and The Blackett Laboratory, Imperial College of Science, Technology, and Medicine, Prince Consort Road, London SW7 2BZ, England

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ABSTRACT: By combining our knowledge of the crystal structure of the glycolytic NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the sequence of the photosynthetic NADP-dependent GAPDH of the chloroplast, two particular amino acid residues were predicted as the principal determinants of differing coenzyme specificity. By use of site-directed mutagenesis, the amino acids Leu 187 and Pro 188 of GAPDH from *Bacillus stearothermophilus* have been replaced with Ala 187 and Ser 188, which occur in the sequence from the chloroplast enzyme. The resulting mutant was shown to be catalytically active not only with its natural coenzyme NAD but also with NADP, thus confirming the initial hypothesis. This approach has not only enabled us to alter the coenzyme specificity by minimal amino acid changes but also revealed factors that control the relative affinity of the enzyme for NAD and NADP.

The NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[1] (EC 1.2.1.13) from chloroplasts, which is involved in photosynthetic assimilation of CO_2 through the Calvin cycle (McGowan & Gibbs, 1974; O'Brien & Powls, 1976; Pupillo & Fagiani, 1979), has been shown to be closely homologous with the glycolytic enzyme from thermophilic eubacteria (EC 1.2.1.12), from which it has been proposed to have derived (Martin & Cerff, 1986). However, the thermophilic enzyme is strictly NAD-dependent, in contrast to the chloroplast enzyme, which functions with both coenzymes NAD and NADP but with a preference for NADP (Ferri et al., 1978; Cerff, 1978). The dual specificity of the chloroplast enzyme contrasts with that of many other NAD/NADP-dependent dehydrogenases, which have a marked preference for a specific coenzyme (Stryer, 1988).

Analysis of coenzyme binding in several dinucleotide-binding enzymes (Wierenga et al., 1985) showed that there is a structural homology within a $\beta\alpha\beta$ folding unit to which the ADP moiety of the coenzyme binds in a similar fashion. This fingerprint region contains amino acid sequences that are characteristic for NAD or NADP binding (Rice et al., 1984). Recently Scrutton et al. (1990) redesigned the coenzyme specificity of NADP-dependent glutathione reductase by directed mutagenesis and confirmed the set of amino acid residues that are determinants of differing coenzyme specificity.

The three-dimensional structures of both the holo and apo forms of *Bacillus stearothermophilus* GAPDH have been determined at high resolution (Skarzynski et al., 1987; Skarzynski & Wonacott, 1988), and the gene for this enzyme has been cloned, expressed, and sequenced (Branlant et al., 1983, 1989). The coenzyme-binding domain of GAPDH contains an ADP-binding fingerprint typical of an NAD-dependent enzyme. However, the chloroplast enzyme does not show the expected sequence differences for NADP specificity, suggesting that a different mechanism is involved in the molecular recognition of NADP in GAPDH.

We have used the GAPDH structures together with the primary sequences of chloroplast enzymes to explore the molecular basis for coenzyme specificity using molecular modeling. Amino acids have been replaced by site-directed mutagenesis, and our predictions have been tested by measurements of the properties of the mutant enzyme.

Materials and Methods

Molecular modeling was carried out on an Evans & Sutherland graphics system PS300 using the program FRODO (Jones, 1978).

(*a*) *Mutagenesis and Isolation of Mutant Enzyme.* To produce the double mutant, a 34-base oligonucleotide was synthesized (5′ CGCAAATCTTTATGGGAAGCGTCCAAAATTCGTT 3′) with five mismatches as compared to the wild-type sequence (Branlant et al., 1989). The method used to generate the mutated enzyme was that previously described (Mougin et al., 1988; Soukri et al., 1989). The gene was then totally sequenced by the dideoxy method (Sanger et al., 1977) to verify that no other mutation had arisen. Production and purification of the wild-type and mutant enzymes were per-

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* To whom correspondence should be addressed.
‡ Laboratoire d'Enzymologie et de Gênie Gênêtique.
§ The Blackett Laboratory.

[1] Abbreviations: GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); G3P, glyceraldehyde 3-phosphate; 1,3-dPG, 1,3-diphosphoglycerate; P_i, inorganic phosphate; NAD and NADH, nicotinamide adenine dinucleotide (oxidized and reduced forms); NADP and NADPH, nicotinamide adenine dinucleotide phosphate (oxidized and reduced forms); 3-CAPAD, 3-(chloroacetyl)pyridine adenine dinucleotide.

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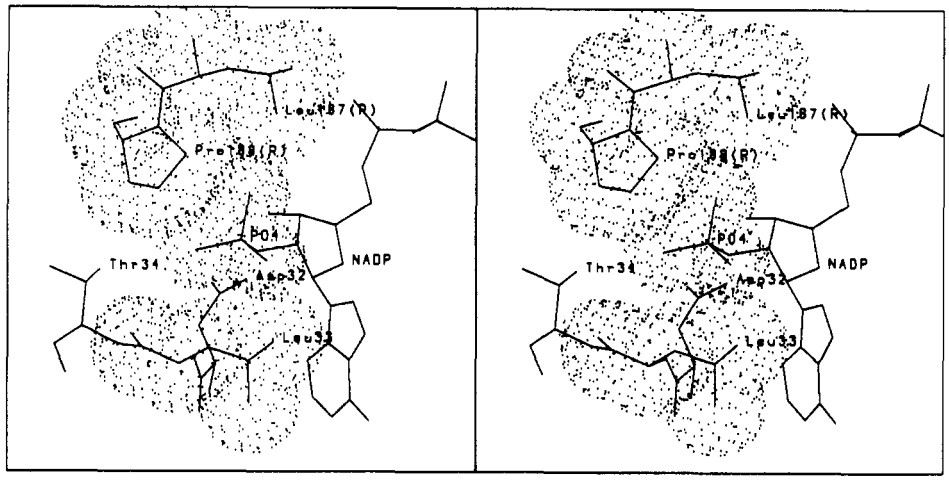

FIGURE 1: Stereoview of binding site for the adenosine moiety of NAD in the holoenzyme structure of *B. stearothermophilus* GAPDH. The van der Waals surface is shown for the 2′-phosphate of NADP and the adjacent residues. The position determined for the 2′-phosphate of NADP from model building indicates that it is in close contact with residues Leu 187 and Pro 188 from the S-loop of the adjacent related subunit as well as Leu 33.

formed as described earlier (Mougin et al., 1988).

(*b*) *Enzyme Assays.* Enzymic activity was measured on a Cary spectrophotometer, following appearance or disappearance of NAD(P)H at 340 nm. Initial rate measurements were performed according to Ferdinand (1964) for oxidative phosphorylation (40 mM triethanolamine, 0.2 mM EDTA, and 50 mM phosphate, pH 8.9) and with 10 mM PIPES buffer, pH 7.1, for reductive dephosphorylation. All Michaelis substrate values were determined at saturating concentrations of other ligands. Michaelis and inhibition constants were determined by Lineweaver–Burk double-reciprocal plots or Dixon plots.

(*c*) *Inactivation of GAPDH by 3-CAPAD.* Inactivation of wild-type and mutant GAPDH (0.17 μM) was studied at 22 °C in 0.1 M phosphate buffer, pH 8.5. The concentration of 3-CAPAD was varied over the range 6–55 μM for wild type and 5–70 μM for the mutant. Aliquots were withdrawn from the incubation mixture at fixed intervals and added directly to the assay mixture. The protective effect of NAD(P) on the inactivation rate was studied at 52 and 70 μM 3-CAPAD for the wild-type and the mutant enzymes, respectively. The resulting curves were interpreted according to Kitz and Wilson (1962), Meloche (1967), and Branlant et al. (1982).

(*d*) *Thermal Inactivation.* Thermal inactivation experiments were carried out by incubating a solution of either wild-type or mutant apoenzyme (1 mg/mL) in 20 mM Tris and 2 mM EDTA, pH 8, over a temperature range from 40 to 90 °C. Parallel experiments were performed with saturating concentrations of NAD (2 mM) or NADP (20 mM). After 10 min of incubation, aliquots were cooled in an ice bath and residual activity was determined as described above.

RESULTS

Sequence Comparison. Comparison of the primary structure of the chloroplast and the glycolytic enzymes reveals a very high degree of sequence identity (Martin & Cerff, 1986): *B. stearothermophilus* and maize chloroplast GAPDHs are 60% identical (Branlant et al., 1989; Quigley et al., 1988). In particular, all the amino acids that are directly implicated in the catalytic mechanism and substrate specificity are conserved.

In searching for the possible causes of changed coenzyme specificity in the chloroplast enzyme, we have examined the amino acid sequence differences in the region close to the binding site for the adenosine moiety of NAD in the model

Table I: Amino Acid Sequence Alignments

(A) The S-Loop Region (Residues 178–200) of GAPDH[a]			
	180	190	200
B. stearothermophilus	YTNDQR	ILDLP-HKDLRRARAAA	
maize or mustard chloroplast	G L	AS- R	
(B) Peptide 30–50[b]			
	30	40	50
B. stearothermophilus	VNDLT	-DANT L A HLLKYDS	VH
maize chloroplast	I	TG-GVKQA S	T L

[a] The *B. stearothermophilus* GAPDH sequence is from Branlant et al. (1989), the maize chloroplast GAPDH sequence is from Quigley et al. (1988), and the mustard chloroplast GAPDH sequence is from Martin and Cerff (1986). [b] Residues 32–35 form a loop between β-sheet B and α-helix C (residues 36–45). The numbering of amino acid residues is according to Biesecker et al. (1977).

of the *B. stearothermophilus* holoenzyme. The structure of *B. stearothermophilus* GAPDH shows that residues 178–200 form the S-loop region, which is at the core of the tetramer, and that amino acid side chains of Leu 187 and Pro 188 lie close to the adenine ribose of the *R*-axis related subunit (Figure 1). For this particular part of the sequence, the enzyme from thermophilic sources is characteristically different from the enzyme from mesophiles (Walker et al., 1980). The chloroplast enzyme shows only conservative changes (Table I) apart from residues at positions 187 and 188, where Leu and Pro are replaced by Ala and Ser. This double sequence change could easily be accommodated within the structure, since it involves no more than the deletion of side-chain atoms in hydrophobic contacts with other parts of the molecule and would not be expected to perturb the secondary or tertiary structure of the enzyme.

One initially surprising feature of the sequence of the chloroplast enzyme is the conservation of the negatively charged residue Asp 32, which in common with other NAD-binding sites is directly involved in the specific binding of the adenosine ribose through hydrogen bonds with both hydroxyl groups of the ring. Replacement of NAD by NADP as coenzyme, which involves the addition of a phosphate group at the 2′-position of the ribose ring, would bring together these two negatively charged groups and create an energetically

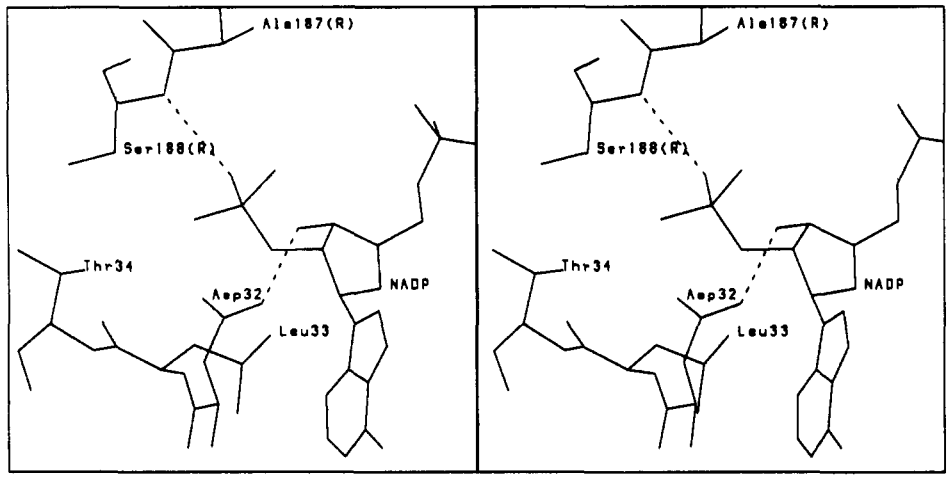

FIGURE 2: Stereoview of the proposed binding of NADP to the mutant enzyme with Leu 187 Ala and Pro 188 Ser. Note the hydrogen bond from the main-chain NH of Ser 188 to the 2′-phosphate of NADP. A water molecule (not shown) can be accommodated in the space previously occupied by the proline and leucine side chains.

unfavorable interaction. However, an important feature of the structure is the presence of hydrogen bonds to the carboxylate group of Asp 32 from the main-chain NH groups at the carboxy-terminal ends of the adjacent strands of β-sheet that make up the $\beta\alpha\beta$ motif of the ADP-binding fold (Wierenga et al., 1985). For the apoenzyme, the NH groups involved are from residues 8 and 34, while for the holoenzyme, as a consequence of the coenzyme-binding domain movement and the accompanying conformational change of the main-chain residues 33–36 (Skarzynski & Wonacott, 1988), the hydrogen bond switches from the peptide NH of residue 34 to that of 33. Thus the carboxylate of Asp 32 is not only held in place by hydrogen bonds from the ends of the β-sheet but also changed in character, since in the presence of a bound ribose group with a full complement of hydrogen bonds the negative charge of the carboxyl group will be delocalized and effectively neutralized. Thus the energetic penalty due to repulsion of like charges when NADP replaces NAD as coenzyme will be much reduced. Nonetheless, with NADP bound the loss of the potential hydrogen bond to the 2′-hydroxyl of the ribose will require an alternative hydrogen-bond proton donor if this is not to result in reduced affinity for NADP.

Molecular Modeling. In our initial model-building study the additional 2′-phosphate group of NADP was added with appropriate geometry to the *B. stearothermophilus* model of NAD bound to the protein in the holoenzyme structure. Although the adenine ribose is partially solvent accessible, the phosphate group is restricted stereochemically to a position in which it is in close contact with Leu 33 and with Pro 188 and Leu 187 from the *R*-axis related subunit (see Figure 1). The hydrophobic environment would be most unfavorable for the binding of the phosphate group and would mitigate against binding of NADP to the holo form of the enzyme. The substitution of Leu 187 and Pro 188 by the side chains of alanine and serine not only relieves the steric hindrance with the 2′-PO_4 but also provides a more hydrophilic site in which hydrogen bonds can be formed with the serine hydroxyl or main-chain NH, which would lead to increased specificity for NADP (see Figure 2). In other NADP-dependent enzymes, such as glutathione reductase (Scrutton et al., 1990), the binding site for the 2′-PO_4 contains two arginine residues, which neutralize the charge and provide specificity. There are no charges in sequence for the chloroplast enzyme that could provide a similar interaction, and none of the existing arginine or lysine residues can be brought close enough to the 2′-phosphate for a direct interaction to occur. Thus the dual specificity cannot arise through a switch of side-chain conformation of positively charged residues.

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

substrate or inhibitor	K_m (10^{-3} M)	K_i[b] (10^{-3} M)	k_{cat}[c] (s^{-1})
(A) Wild-Type (*B. stearothermophilus*) GAPDH			
NAD	0.15 ± 0.02	0.10 ± 0.01	280
G3P	0.8 ± 0.1		
P_i	18 ± 5		
1,3-dPG	0.016 ± 0.005		420
NADH	0.012 ± 0.003		
NADP		2 ± 0.1	
(B) Mutant (Leu 187 Ala, Pro 188 Ser) GAPDH			
NAD	0.35 ± 0.05	0.20 ± 0.02	280
G3P	1.5 ± 0.2		
P_i	33 ± 5		
NADP	7.1 ± 0.6	1.0 ± 0.1	58
G3P	0.15 ± 0.01		
P_i	400 ± 10		
NADH	0.040 ± 0.006		290
1,3-dPG	0.033 ± 0.005		
NADPH	0.34 ± 0.06		35
1,3-dPG	0.032 ± 0.005		

[a] The reaction was performed at 22 °C in 40 mM triethanolamine and 0.2 mM EDTA buffer, pH 8.9, for the oxidative phosphorylation and in 10 mM PIPES buffer, pH 7.1, for the reductive dephosphorylation. Each value represents the average of three determinations. [b] K_i values were determined by using NADH as the varied coenzyme. [c] Variations of the k_{cat} values were below 10%. Note that the pH–rate profiles in the forward and reverse reactions were found to be similar for wild-type and mutant GAPDH, using NAD(H) as coenzyme. Similar pH–rate profiles were also observed for the mutant enzyme, using NADP(H) as coenzyme (curves not shown).

We also examined the possibility of binding NADP to the native apoenzyme, using the position of bound ADP (unpublished results) as a starting model. In this "open" conformation state of the enzyme there is no apparent steric hindrance to the addition of a phosphate group to the 2′-hydroxyl of the ribose ring. However, the presence of this phosphate would interfere with the normal conformational changes associated with coenzyme binding, which leads to the "closed" holoenzyme state.

Properties of the Mutant Enzyme. The catalytic properties of the purified mutant enzyme are compared with those of the wild-type enzyme in Table II. The mutations have very little effect on the kinetic parameters of the enzyme using NAD(H)

Table III: Dissociation Constants of 3-CAPAD, NAD, and NADP for Wild-Type and Mutant GAPDH from *B. stearothermophilus*[a]

	K_i (μM)		k (min^{-1})	
	wild type	mutant	wild type	mutant
3-CAPAD	11 ± 1	28 ± 2	0.33 ± 0.04	0.24 ± 0.03
NAD	0.8 ± 0.1	2.8 ± 0.2		
NADP	1100 ± 180	350 ± 30		

[a] The reaction was performed at 22 °C in 0.1 M phosphate buffer, pH 8.5. Experimental conditions: GAPDH, 0.17 μM; 3-CAPAD, 6–55 and 5–70 μM for wild-type and mutant enzyme, respectively. For more details, see Materials and Methods. In all cases, the 3-CAPAD inactivation process exhibited pseudo-first-order kinetics until more than 80% of the GAPDH activity was lost. Inactivation rate constants (k) and dissociation constants (K_i) of 3-CAPAD were deduced from curves representing the double-reciprocal plot of 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.

as the coenzyme (Table II). This strongly suggests that the mode of NAD binding is unaffected by the mutations, including the precise positioning of the nicotinamide ring. This point was confirmed by examining the "Racker band" (Racker & Krimsky, 1952), which is sensitive to changes at the active site. Mougin et al. (1988) showed that the formation of a charge-transfer transition in GAPDH involved the essential residue Cys 149 and the pyridinium ring of NAD, acting as an electron donor and electron acceptor, respectively. The Racker band was found to be the same for the wild-type and the mutant enzyme (ϵ_{360} = 1100 M^{-1} cm^{-1}; curves not shown), indicating that no significant change has occurred in the location and orientation of the nicotinamide group in the active site.

With the mutant enzyme the values of K_m for NAD and NADH are increased 2.3-fold and 3.3-fold, respectively (see Table II). To test whether this is a direct consequence of a lower affinity for both coenzymes, two additional experimental investigations were carried out. First, the inhibition constant K_i for NAD can be determined by using 3-(chloroacetyl)-pyridine adenine dinucleotide (3-CAPAD) as an affinity labeling probe by measuring the protection against inactivation afforded by the coenzyme (Branlant et al., 1982). NAD dissociation constants of 0.8 μM and 2.8 μM were determined at pH 8.5 for the wild-type and mutant GAPDH, respectively (Table III). As already observed (Branlant et al., 1982), these values correspond closely to those of the NAD-binding sites of low affinity. Second, an inhibition constant for NAD can be determined from steady-state rate measurements, with NAD acting as a competitive inhibitor against NADH in the reaction with 1,3-dPG. As shown in Table II, K_i values of 0.10 mM and 0.20 mM were determined for the wild-type and mutant enzymes. The apparent discrepancy in the two sets of K_i values can be explained by the fact that in the former experiment K_i is a measure of the affinity of NAD for the apoenzyme, while in the latter K_i would represent the affinity of NAD for the acyl enzyme intermediate (Malhotra & Bernhard, 1989). In both instances the affinity of NAD for the mutant GAPDH is reduced by a factor of 2 to 3 relative to that for the wild-type enzyme. This difference in affinity is also observed for 3-CAPAD where dissociation constants of 11 μM and 28 μM were determined for the wild-type and the mutant enzymes, respectively (Table III).

Our prediction of the crucial role of residues Leu 187 and Pro 188 in affecting productive NADP binding was confirmed in that the mutant was found to be catalytically functional with NADP as a coenzyme (Table IIB). However, the kinetic properties of the mutant enzyme with NADP(H) are markedly

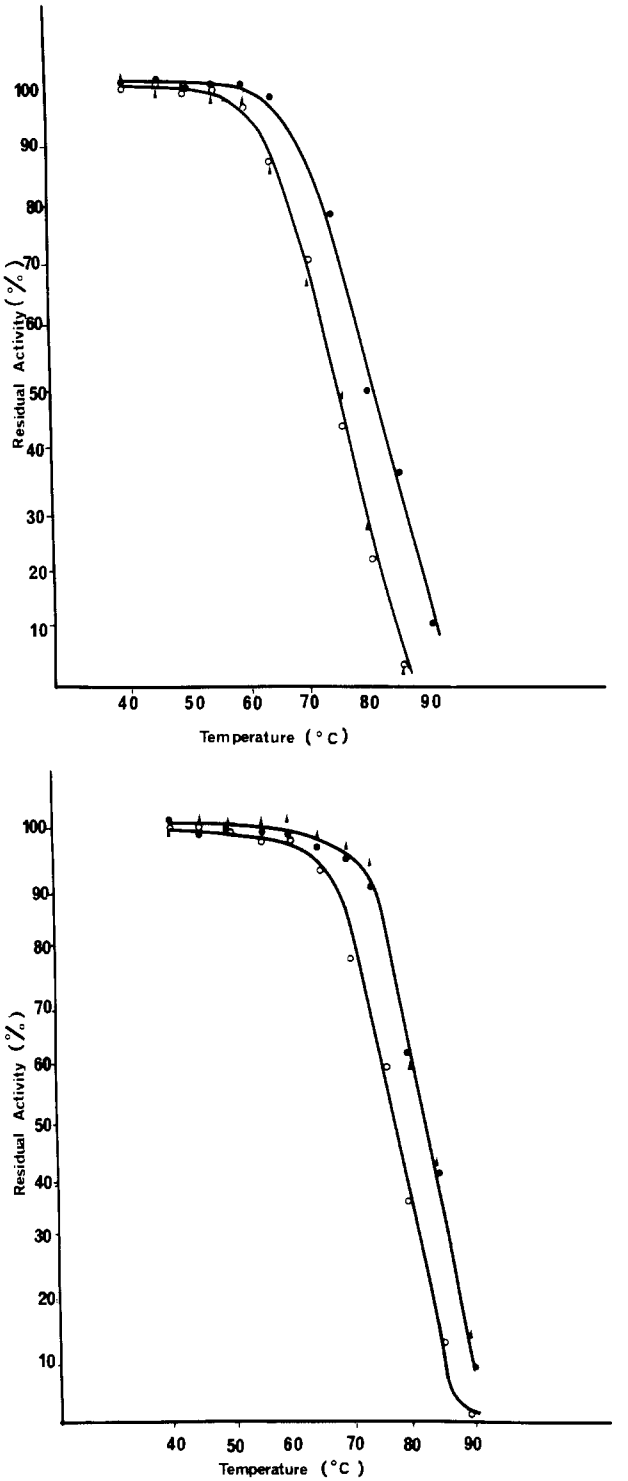

FIGURE 3: Thermal inactivation of *B. stearothermophilus* GAPDH: (○) apoenzyme, (●) enzyme with 2 mM NAD, and (▲) enzyme with 20 mM NADP. (a) Wild-type enzyme; (b) mutant enzyme. 100 μL of enzyme solutions (1 mg/mL in 20 mM Tris and 2 mM EDTA, pH 8) was heated for 10 min at various temperatures and cooled in ice. The remaining enzyme activity was then determined as described under Materials and Methods.

different from those with NAD(H). First, the K_m values for the phosphorylated coenzymes are increased 20-fold relative to that for NAD and 9-fold relative to that for NADH. Second, the enzyme activity is reduced with both G3P and 1,3-dPG. Third, while both NAD and NADP protect the mutant enzyme against thermal inactivation to the same degree, only NAD protects the wild-type GAPDH (Figure 3), implying that protection is associated with the presence of

catalytic activity and must involve the apo–holo structural transition. The presence of an equivalent Racker band with NADP (curve not shown) also suggests that the nicotinamide moiety is bound equivalently in the active site, and differences, if any, must be confined to the adenosine portion of the coenzyme. Finally, the dissociation constants of NADP for the mutant and wild-type enzyme were determined as 0.35 mM and 1.1 mM, respectively, by 3-CAPAD titration (Table III), while steady-state kinetic measurements with NADH as coenzyme provide values of K_i for NADP of 1 mM for the mutant and 2 mM for the wild-type enzyme (Table II). Both experiments show that NADP binds to the wild-type enzyme, even though it is unable to play a catalytically functional role, while the mutation increases the affinity for NADP, by a factor of 2 to 3 depending on the state of the enzyme.

At present, we are unable to account for the large charges in the substrate K_m values for G3P and inorganic phosphate (P_i) with NADP as coenzyme (see Table IIB), but similar properties are observed for the chloroplast enzyme (Ferri et al., 1978).

Discussion

Studies using NAD as the coenzyme show that the mutant behaves essentially the same as the wild-type enzyme except that K_i and K_m are both increased. This can almost certainly be ascribed to the loss of hydrophobic contacts between NAD and the side chain of Leu 187. The properties of the mutant enzyme with NADP as coenzyme require more explanation. The larger values of K_i and K_m for NADP relative to NAD are not surprising since the enforced proximity of the 2′-phosphate of NADP to the carboxylate of Asp 32 will not be fully compensated and the carboxyl group will have one unsatisfied hydrogen bond. The 4-fold lower k_{cat} with NADP could be due to the additional phosphate interfering with the full conformational change from the apo structure, in which the subunit is in an open conformation allowing coenzyme binding, to the holo structure, in which the coenzyme is almost buried. This conformational change is responsible for optimizing the relative disposition of the catalytic groups and ion-binding ligands in the active site (Skarzynski & Wonacott, 1988). Indeed, the weak inhibitory effect of NADP on the catalytic activity of yeast GAPDH (Yang & Deal, 1969) and the K_i measurements for *B. stearothermophilus* (Table IIA) show that NADP binds weakly to GAPDH but cannot attain a catalytically active state. The equivalent Racker band with NADP and NAD and the similar thermal protection for NAD and NADP both argue that NADP binding to the mutant induces a full conformational transition that leads to a fully active holoenzyme state. The fact that the K_m and K_i of the mutant enzyme for NADP are higher than those of the chloroplast GAPDH by about 2 orders of magnitude (Ferri et al., 1978; Cerff, 1978) indicates a tighter binding of NADP to the chloroplast enzyme than to the mutant GAPDH.

Inspection of the model of the *B. stearothermophilus* enzyme shows that residues Asp 32, Leu 33, and Thr 34 contribute to the formation of the adenosine-binding site (Skarzynski & Wonacott, 1988). During the binding of NAD, the loop consisting of residues 33–36 changes conformation so that Leu 33 moves into contact with the adenine ring. In the chloroplast enzyme sequences, Leu 33 is replaced by a threonine and the resulting favorable side-chain conformation places the hydroxyl group close to the 2′-phosphate of NADP, while the side chain maintains a hydrophobic contact with the adenine ring. This change is likely to further enhance the binding of NADP.

Other differences between the chloroplast and glycolytic GAPDHs have been examined. The model of the *B. stearothermophilus* structure shows that removal of the side chains of Thr 34 and Asp 36 by replacement with glycines and the change of Leu 40 to alanine would, in the absence of a conformational change, create a hole adjacent to residue Asp 32, which would be accessible to the solvent. This would permit hydrogen-bond formation with the carboxylate oxygen to which the O2′ of the ribose of NAD is normally hydrogen bonded. In addition, the removal of the side chains of Thr 34 and Asp 36 not only gives the peptide loop 32–36 more flexibility but also leaves a space that could be filled by movement of the loop during NADP binding. Further mutagenesis studies are underway to confirm these points.

In summary, by combining the knowledge of the sequence of the chloroplast enzyme with the three-dimensional structure of *B. stearothermophilus* GAPDH, it has been possible to predict the principal residues responsible for the altered coenzyme specificity. This has been confirmed by the use of site-directed mutagenesis. The mutated GAPDH from *B. stearothermophilus* (from Leu 187 and Pro 188 to Ala 187 and Ser 188) has been shown to be capable of catalysis not only with its natural coenzyme NAD but also with NADP. We have therefore been able to confer dual specificity by minimal changes in protein sequence and with little change in the properties of the enzyme with NAD. The site for binding of the 2′-phosphate contrasts with that of the NADP-specific enzymes in having no positively charged residues associated with the phosphate group; instead the phosphate is bound within a hydrophilic pocket formed from groups on the protein that provide hydrogen-bonding donors.

The ability to alter coenzyme specificity is likely to be of value for NAD-dependent dehydrogenases of industrial importance, such as formate dehydrogenase (Hummel & Kula, 1989), since that would provide an effective route for regeneration of NADPH in addition to NADH.

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Registry No. NAD, 53-84-9; NADP, 53-59-8; NADH, 58-68-4; NADPH, 53-57-6; G3P, 591-57-1; 1,3-dPG, 1981-49-3; P_i, 14265-44-2; 3-CAPAD, 39938-03-9; L-Leu, 61-90-5; L-Pro, 147-85-3; L-Ala, 56-41-7; L-Ser, 56-45-1; NAD-dependent GAPDH, 9001-50-7; NADP-dependent GAPDH, 37250-87-6.

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Photochemically Modified Myeloperoxidase, with Optical Spectral Properties Analogous to Those of Lactoperoxidase, Retains Its Original Catalytic Activity†

Hiroshi Hori
Department of Biophysical Engineering, Faculty of Engineering Science, Osaka University, Toyonaka, Osaka 560, Japan
Masao Ikeda-Saito*
Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106
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ABSTRACT: During the course of a reducing reaction using ketyl radicals generated from ketone photoreduction with ultraviolet light, a photoinduced chemical modification of the chromophore group in myeloperoxidase has been found. Light absorption and resonance Raman spectra for this modified enzyme indicated an iron porphyrin chromophore group. The alkaline pyridine hemochrome of the modified enzyme exhibited an optical spectrum closely related to that of iron protoporphyrin IX. The chromophore group of the modified myeloperoxidase was cleaved from the protein by methoxide. Proton magnetic resonance of the diamagnetic bis(cyanide) compound of the extracted heme group showed the presence of two vinyl and three methyl side chains associated with a porphyrin macrocycle. These data provide further insight into the structure of the active site in myeloperoxidase. The EPR spectral properties and enzymatic activities of the native myeloperoxidase are essentially conserved in the modified enzyme. Our present results indicate that the heme peripheral substituent is modified while the stereochemical structure surrounding the chromophore group is not altered by the photochemical modification.

Myeloperoxidase is a major component of the antimicrobial system of polymorphonuclear neutrophils (Klebanoff & Clark, 1978). One of the unique properties of myeloperoxidase is its ability to catalyze the formation of hypochlorous acid from hydrogen peroxide and chloride ion (Harrison & Schultz, 1976). Despite many investigations of the spectroscopic and enzymatic properties of myeloperoxidase (Schultz, 1980), the chemical structure of the heme group in this enzyme has not so far been identified because of the inability to extract the heme prosthetic group from the enzyme protein by usual methods, possibly due to the covalent linkage between the heme group and the apoprotein. Nevertheless, several proposals for the chemical structure of the heme group of this enzyme have appeared in the last two decades. Myeloperoxidase, in its reduced form, has a strong absorption band far to the red of α-bands of other reduced heme proteins containing iron protoporphyrin IX. This unusual spectral property is very similar to that of sulfmyoglobin (Nicholls, 1961). It thus seemed that the iron center in myeloperoxidase was a sulfheme or an iron chlorin (Newton et al., 1965a,b). From the reaction of carbonyl reagents with myeloperoxidase, it was suggested that this enzyme might have a formyl substituent in the heme prosthetic group (Harrison & Schultz, 1978; Wu & Schultz, 1975; Odajima, 1980). The spectrum of the alkaline pyridine hemochrome formed from myeloperoxidase was very similar to that of heme *a* obtained from cytochrome oxidase, which contains a formyl group at the 8-position and a vinyl group at the 4-position; thus at least two conjugated electrophilic groups on opposite pyrroles could be considered, implying that the iron complex of 2,4-divinyl-8(5)-formyldeuteroporphyrin IX was the heme of myeloperoxidase (Wu & Schultz, 1975; Harrison & Schultz, 1978). Recent magnetic circular dichroism and resonance Raman scattering studies (Eglinton

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* To whom correspondence should be addressed.

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