A Highly Active Thermophilic Semisynthetic Flavoenzyme

Donald Hilvert,[†] Yasumaru Hatanaka,[‡] and E. T. Kaiser*

Contribution from the Laboratory of Bioorganic Chemistry and Biochemistry, The Rockefeller University, 1230 York Avenue, New York, New York 10021. *Received January 26, 1987*

Abstract: A thermophilic semisynthetic flavoenzyme has been prepared by alkylation of Cys-149 at the active site of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Bacillus stearothermophilus* with 7-(α-bromoacetyl)-10-methylisoalloxazine. The initially formed tetrameric flavoprotein irreversibly dissociates into dimers. Dimeric 7-acetylflavo-GAPDH is very stable and serves as a catalyst for the oxidation of 1,4-dihydronicotinamides at temperatures as high as 55 °C. NADH is the best substrate examined with the enzyme. Under aerobic conditions at 25 °C the oxidation of this compound is characterized by apparent negative cooperativity, but at low concentrations of NADH a ca. 6000-fold rate acceleration is observed over the analogous model reaction catalyzed by 7-acetyl-10-methylisoalloxazine. The rate of turnover of the enzyme is partially limited by the relatively slow reoxidation of the enzyme-bound dihydroflavin at the active site by molecular oxygen. The efficiency of the actual hydride-transfer step was therefore estimated with anaerobic stopped-flow techniques. In the presence of a large excess of NADH, reduction of the enzyme-bound flavin is characterized by biphasic kinetics with roughly half the flavins reduced in a very rapid step. Thus, bacterial 7-acetylflavo-GAPDH exhibits "half-of-the-sites" reactivity, a classic manifestation of negative cooperativity. Saturation kinetics are found for the rate constant for the fast phase, giving a maximum value of k_{obsd} of 1.14 s^{-1} and a K_m of 12.4 μM. The selectivity of the semisynthetic enzyme was probed by examining its ability to oxidize chirally deuteriated NADH derivatives as well as *N*-alkyl-1,4-dihydronicotinamides. The observed substrate specificity and stereoselectivity can be understood in terms of the postulated dimeric structure of the flavoenzyme.

We are interested in the rational design of biomimetic catalysts and have explored an approach toward catalyst design involving "chemical mutation" of existing enzyme active sites.[1] Tertiary structures present in naturally occurring proteins provide an excellent starting point for the construction of artificial enzymes, since they bind a wide range of potential substrates under mild aqueous conditions. Ideally, covalent attachment of a foreign catalytic group to a specific amino acid residue in a protein binding pocket yields a hybrid molecule that combines the characteristic reactivity of the prosthetic group with the binding specificity of the template.

We have recently used this strategy to prepare a family of semisynthetic flavoenzymes based on the tetrameric template glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from rabbit muscle.[2] The oxidation of NADH, the best substrate for these catalysts, was substantially accelerated relative to an appropriate nonenzymatic model reaction and occurred with *si*-face stereoselectivity. The observed stereoselection and substrate specificity had been predicted from a priori considerations of active-site geometry and were opposite that found for another semisynthetic enzyme, flavopapain, previously prepared in our laboratory, which shows a preference for *N*-alkyl-1,4-dihydronicotinamides. Unfortunately, the detailed characterization of 7-acetylflavo-GAPDH was hampered by the limited stability of the template protein.

Unlike its counterpart from rabbit muscle, GAPDH from the thermophilic bacterium *Bacillus stearothermophilus* is known to be exceptionally stable to both heat and urea.[3] Its primary[4] and tertiary[4,5] structures have been determined, and the protein is highly homologous with the muscle enzymes.[6] We have now isolated this enzyme from bacterial cell paste and used it for the formulation of semisynthetic flavoenzymes. We report here the preparation and properties of one particularly active, thermophilic flavo-GAPDH.

Experimental Section

Instrumentation. UV-vis spectra were measured on a Perkin-Elmer λ-5 spectrophotometer equipped with a thermostated cell holder. Fluorescence spectra were recorded on a Perkin-Elmer 650-40 fluorescence spectrophotometer. Rapid mixing experiments were carried out on a Durrum-Gibson stopped-flow spectrophotometer thermostated at 25 °C. High-performance liquid chromatography (HPLC) was performed with a Beckman Model 344 chromatography system equipped with a Model 164 variable-wavelength detector. NMR spectra were obtained on a Nicolet NT-360 spectrometer (360 MHz). Computer graphics were obtained with the interactive graphics language GRAMPS[7] and the molecular modeling program GRANNY.[8] The solvent accessible surface of GAPDH was calculated by using the program MS.[9]

Materials. Rabbit muscle GAPDH was purchased from Boehringer Mannheim and used without further purification. *B. stearothermophilus* cell paste was obtained from the Centre for Applied Microbiology and Research (Porton, Wilts, England), and GAPDH was isolated[10] from it and purified[3,11] according to literature procedures. NADH and NADPH were purchased from Sigma. Deuteriated dihydronicotinamides were synthesized from NADH by literature procedures.[12] The preparation of *N*-alkyl-1,4-dihydronicotinamides has been previously described.[13] Other materials were of the highest quality commercially available and were used without further purification.

GAPDH Modification. Prior to alkylation, rabbit muscle GAPDH was treated with acid-washed Norit to remove bound NAD^+.[14] Freshly prepared apoenzyme (1.5×10^{-5} M in aqueous buffer, pH 7.5) was then mixed at room temperature with a 12-fold excess of 7-(α-bromoacetyl)-10-methylisoalloxazine[13] (**1a**) (30 mM stock in DMSO). After incubation for 30 min, a 5-fold molar excess of cysteine was added,[13] followed by a second addition of a 9-fold excess of the brominated isoalloxazine. After an additional 20 min, flavo-GAPDH was separated from unreacted **1a** by gel filtration on Sephadex G-25 (2 × 45 cm) with 50 mM EPPS buffer, pH 7.5 at 4 °C. The alkylated rabbit muscle enzyme was unstable and therefore characterized immediately following isolation. Enzyme solutions were generally discarded after 1 day.

Holo-GAPDH from *B. stearothermophilus* (40 mg) was chromatographed on a column of immobilized Cibacron blue (Pierce, 2.5 × 4 cm) to remove bound NAD^+.[15] The resin was washed with 200 mL of 10

[†] Current address: Department of Molecular Biology, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, CA 92037.

[‡] Current address: Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, 060 Japan.

(1) Kaiser, E. T.; Lawrence, D. S. *Science (Washington, D.C.)* **1984**, *226*, 505-511.
(2) Hilvert, D.; Kaiser, E. T. *J. Am. Chem. Soc.* **1985**, *107*, 5805-5806.
(3) (a) Suzuki, K.; Harris, J. I. *FEBS Lett.* **1971**, *13*, 217-220. (b) Amelunxen, R. E. *Biochim. Biophys. Acta* **1967**, *139*, 24-32.
(4) Biesecker, G.; Harris, J. I.; Thierry, J. C.; Walker, J. E.; Wonacott, A. J. *Nature (London)* **1977**, *266*, 328-333.
(5) Leslie, A. G. W.; Wonacott, A. J. *J. Mol. Biol.* **1984**, *178*, 743-772.
(6) Harris, J. I.; Waters, M. In *The Enzymes*; Boyer, P., Ed.; Academic: New York, 1976; Vol. XIII, Part C, pp 1-49.
(7) O'Donnell, T. J.; Olson, A. J. *Comput. Graphics* **1981**, *15*, 133-142.
(8) Connolly, M. L.; Olson, A. J. *Comput. Chem.* **1985**, *9*, 1-6.
(9) Connolly, M. L. *Science (Washington, D.C.)* **1983**, *221*, 709-713.
(10) Atkinson, T.; Banks, G. T.; Bruton, C. J.; Comer, M. J.; Jakes, R.; Kamalagharan, T.; Whitaker, A. R.; Winter, G. P. *J. Appl. Biochem.* **1979**, *1*, 247-258.
(11) Harris, J. I.; Hocking, J. D.; Runswick, M. J.; Suzuki, K.; Walker, J. E. *Eur. J. Biochem.* **1980**, *108*, 535-547.
(12) Arnold, L. J., Jr.; You, K.; Allison, W. S.; Kaplan, N. D. *Biochemistry* **1976**, *15*, 4844-4849.
(13) Levine, H. L.; Kaiser, E. T. *J. Am. Chem. Soc.* **1978**, *100*, 7670-7677.
(14) Havsteen, B. H. *Acta Chem. Scand.* **1965**, *19*, 1643-1651.
(15) (a) Branlant, G.; Eiler, B.; Wallen, L.; Biellman, J.-F. *Eur. J. Biochem.* **1982**, *127*, 519-524. (b) Thompson, S. T.; Cass, R.; Stellwagen, E. *Anal. Biochem.* **1976**, *72*, 293-296.

0002-7863/88/1510-0682$01.50/0 © 1988 American Chemical Society

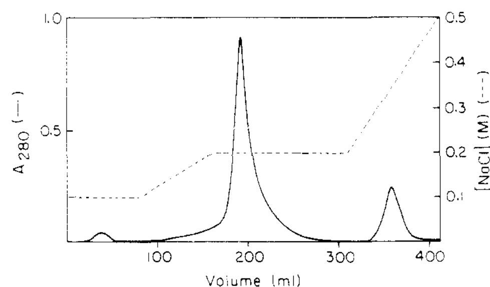


Figure 1. Elution profile of bacterial 7-acetylflavo-GAPDH on Sepharose Q Fast Flow with buffer A and a NaCl gradient. The dimeric flavoenzyme elutes at ca. 200 mL, the tetramer at 360 mL.

mM Tris-HCl (pH 8.5), and the apoenzyme was eluted with 1 M NaCl in the same buffer. The bacterial apoenzyme was treated with bromoflavin analogue **1a** according to the protocol described above for rabbit muscle GAPDH. The resulting flavoenzyme was separated from excess reagent by gel filtration on Sephadex G-25 at 4 °C with buffer A (10 mM Tris-HCl, pH 7.5). The initially isolated tetrameric flavoprotein irreversibly dissociates to give a dimeric species as judged by analytical HPLC on a Toyosoda TSK-DEAE-5PW column (7.5 × 75 mm; 30-min elution gradient, 0–0.4 M NaCl in buffer A; flow rate, 1 mL/min) calibrated with protein standards. After 20 h at 4 °C, the crude flavo-GAPDH mixture was therefore applied to a column of Q-Sepharose Fast Flow (Pharmacia, 1.6 × 10 cm), previously equilibrated with buffer A at 4 °C. Optimal separation of the dimeric and tetrameric forms of bacterial flavoenzyme was achieved with a flow rate of 2 mL/min by controlling the concentration of buffer B (buffer A containing 0.5 M NaCl) as follows: 20% for the first 40 min, 20–40% for 40 min, 40% for 75 min, 40–100% for 50 min (see Figure 1). The two flavoproteins isolated were concentrated by ultrafiltration (Amicon PM-10) and stored at -80 °C. Both fractions retained complete activity for several months under these conditions, although the recovered tetramer was deficient in flavin.

Molecular Weight Estimation. The molecular weights of the various flavo-GAPDH species were estimated by gel filtration. Typically, 1 mL of sample containing 2–3 mg of enzyme was applied to a Sephadex G-150-120 column (1.4 × 100 cm), which had been equilibrated at 4 °C with a pH 7.5 buffer containing 50 mM phosphate, 1 mM EDTA, and 1 mM dithiothreitol. The protein was eluted with this buffer and its elution volume compared with that of protein standards of known molecular weight, including unmodified GAPDH from rabbit muscle and *B. stearothermophilus*. Alternately, gel filtration was performed by HPLC on an LKB column (7.5 × 300 mm of TSK 3000SW; flow rate, 1 mL/min), using 0.02 M phosphate buffer (pH 6.8) containing 0.05 M Na_2SO_4 to elute the enzyme. A mixture of protein standards was used for calibration of this column, as well. The molecular weight of a single subunit of the modified enzyme was determined by electrophoresis on 10% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate as described by Laemmli.[16]

Protein and Flavin Estimation. Protein concentrations were measured by the Biuret method[17] or by amino acid analysis. For the latter, the proteins were hydrolyzed in vacuo in 6 M HCl containing 0.1% phenol and norleucine as an internal standard for 96 h at 110 °C. The hydrolysates were analyzed in a Durrum D-500 analyzer. The results obtained with the two different methods were within 10% of each other. Titration of the flavin chromophore in the alkylated enzymes was performed with sodium dithionite under anaerobic conditions.[18] The oxidized, enzyme-bound isoalloxazine has λ_{max} 432 nm (ϵ 1.04 × 10^4 M^{-1} cm^{-1}). The value of $\Delta\epsilon$ at this wavelength for reduction of the bound flavin was determined to be 8780 ± 200 M^{-1} cm^{-1}.

Enzyme Assays. Native GAPDH activity was measured at 25 °C according to Harris.[11] The oxidoreductase activity of the flavoenzymes was studied, except as noted, at 25 °C in air-saturated buffer (50 mM EPPS, pH 8.0, for muscle enzyme and 10 mM Tris-HCl, pH 7.5, for the bacterial enzyme) in the presence of catalase (0.086 mg/mL) and superoxide dismutase (0.01 mg/mL). The oxidation of the various dihydronicotinamides by **1** was monitored by measuring the decrease in the nicotinamide absorption that occurs between 340 and 360 nm. At low substrate concentrations (0.25–5 μM) the decrease in fluorescence at 460 nm (340 nm for excitation) was determined instead. Initial rates were determined from the first 5 to 10% of reaction for a range of substrate concentrations. The kinetic rate constants were obtained by direct fit of the data to the equations discussed in the text with an iterative nonlinear least-squares computer program.[19]

Rapid Reactions. The rate of reduction of flavo-GAPDH by excess dihydronicotinamide was measured by stopped-flow spectrophotometry under anaerobic conditions. Equal volumes of flavoenzyme and substrate stock solutions were rapidly mixed, and the time-dependent decrease in flavin absorption at 432 nm was followed. Anaerobic conditions were achieved by placing buffer A containing glucose oxidase (0.0025 mg/mL) and catalase (0.086 mg/mL) in a glass vessel and repeatedly evacuating the flask and reequilibrating with argon. Addition of glucose to stock solutions of enzyme (ca. 2.5 μM flavin) and substrate (0.03–2 mM) made with this buffer removed the last traces of oxygen.

Results and Discussion

Modification of Bacterial GAPDH. GAPDH is a tetrameric protein that catalyzes the reversible oxidation of glyceraldehyde 3-phosphate with concomitant reduction of NAD^+.[6] Our preliminary experiments[2] with enzyme isolated from rabbit muscle indicated that it had excellent potential as a template for the construction of semisynthetic flavoenzymes. However, the flavo-GAPDH we prepared proved to be too labile for extensive characterization, mirroring the known[6] relative instability of the unmodified rabbit muscle enzyme. In contrast, GAPDH from the thermophilic bacterium *B. stearothermophilus* is reported[3] to be a very stable species, active after prolonged exposure to urea and at elevated temperatures. Furthermore, the bacterial enzyme is highly homologous to the muscle enzymes in both structure and properties.[3] The three-dimensional structure of the protein refined at 1.8-Å resolution[5] reveals that each subunit has a binding site for NAD^+/NADH and that a reactive sulfhydryl group (Cys-149) is positioned in the vicinity of the pyridinium ring of the bound cofactor (Figure 2a). Model building[7-9] (Figure 2b) convinced us that flavin analogues could be accommodated sterically within the binding site without blocking NAD^+/NADH binding, and we were therefore encouraged to test whether this template would yield stable artificial flavoproteins.

Holo-GAPDH containing three to four molecules of bound NAD^+ was isolated in high yield from *B. stearothermophilus* cell paste.[10] Extracts of the crude enzyme were purified according to the method of Suzuki and Harris.[3a,11] Analysis of the protein using SDS–PAGE on a 10% Laemmli gel[16] showed a single band at M_r 37 000. Since it is known that bound cofactor inhibits alkylation of the active-site sulfhydryl group,[20] NAD^+ was removed from the holoenzyme by chromatography on a Cibacron blue column.[15] The resulting apo-GAPDH was homogeneous as judged by gel electrophoresis and analytical HPLC. Its specific activity was 80 U/mg in a standard assay.[11]

Bacterial apo-GAPDH reacted readily with 7-(α-bromoacetyl)-10-methylisoalloxazine at room temperature in aqueous buffer. The extent of Cys-149 modification was followed by assaying the reaction mixture for the ability to oxidize glyceraldehyde 3-phosphate.[11] After completion of the reaction, residual native activity was less than 1%, and the modified protein was separated from excess reagent by gel filtration. Alkylation was apparently specific for the active-site sulfhydryl group of Cys-149, since the four rapidly titrating thiols of the native tetramer were lost subsequent to modification. As judged by amino acid analysis and dithionite titration[18] of the flavin chromophore, 0.90 ± 0.05 isoalloxazine moiety was incorporated into each subunit of the bacterial enzyme. The absorbance maximum of the enzyme-bound flavin was red-shifted by 5 nm relative to the λ_{max} of 7-acetyl-10-methylisoalloxazine free in solution.

The purity of 7-acetylflavo-GAPDH was determined by analytical HPLC on a Toyosoda TSK-DEAE-5PW ion-exchange column equilibrated with 10 mM Tris-HCl, pH 7.5. The protein was eluted by increasing the NaCl concentration from 0 to 0.4 M in 30 min at a flow rate of 1 mL/min. The initially formed bacterial flavoenzyme **1c** had a retention time of 18.9 min, com-

(16) Laemmli, U. K. *Nature (London)* **1970**, *227*, 680–685.
(17) Cooper, T. G. In *The Tools of Biochemistry*; Wiley-Interscience: New York, 1977; pp 51–53.
(18) Burleigh, B. D., Jr.; Foust, G. P.; Williams, C. H., Jr. *Anal. Biochem.* **1969**, *27*, 536–544.
(19) Yamaoka, K.; Tanigawara, Y.; Nakagawa, T.; Uno, T. *J. Pharmacobio. Dyn.* **1981**, *4*, 879–885.
(20) Trentham, D. R. *Biochem. J.* **1968**, *109*, 603–612.

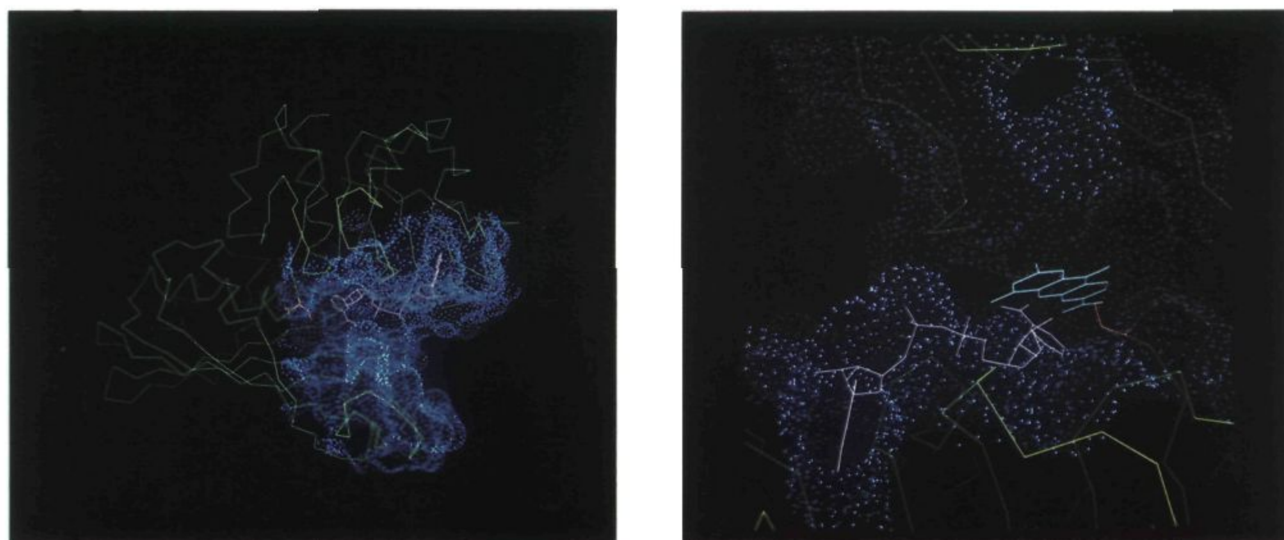
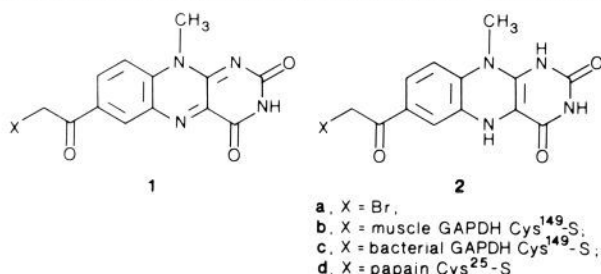


Figure 2. (a, left) One of four identical subunits of holo-GAPDH from the thermophilic bacterium *B. stearothermophilus*. The green ribbon traces the peptide backbone, and the blue dot surface represents the solvent-accessible surface of the active site. The uniquely reactive sulfhydryl group Cys-149 is highlighted in red. Noncovalently bound NAD^+ is shown in magenta. (b, right) Close-up of the active site of GAPDH from *B. stearothermophilus* GAPDH with bound NADH (magenta) with a 7-acetyl-10-methylisoalloxazine moiety (cyan) attached to Cys-149. This crude model indicates that the active site can sterically accommodate a flavin analogue.

pared with 19.3 min for the unmodified apoenzyme. Gel filtration



of this species on Sephadex G-150-120, calibrated with protein standards, gave an estimated value of 164 000 for the molecular weight, identical with that found for the unmodified tetrameric enzyme by the same method. The difference between this value and the actual[6] molecular weight (M_r 146 000) reflects the magnitude of the error inherent in this type of experiment.

Ion-exchange HPLC analysis revealed, however, that the initially formed flavoenzyme undergoes irreversible decomposition on standing in the cold to give a new flavoprotein with a shorter retention time (15.6 min). After 20 h at 4 °C, this new species accounted for more than 80% of total protein injected onto the ion-exchange column. It could be separated from the tetramer on a preparative basis on a column containing the strong anion exchanger Q-Sepharose Fast Flow from Pharmacia. The molecular weight of this new flavoenzyme was determined to be about 76 000 by the gel filtration technique described above. This finding was confirmed by HPLC gel filtration on an LKB 2135 UltraPak TSKG 3000 SW column. Thus, the tetrameric flavoenzyme apparently dissociates into dimers.[21] Since the isolated dimer does not detectibly reassociate, the dissociation process may be

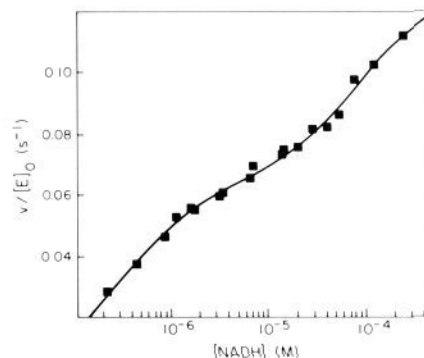


Figure 3. Semilogarithmic plot of the effect of NADH concentration on the rate of its oxidation of 7-acetylflavo-GAPDH in air-saturated buffer (50 mM Tris-HCl, pH 7.5) at 25 °C. The initial rate data (■) were fitted to eq 1. For the experiment depicted, $k_p = 0.0656 \pm 0.0015\ s^{-1}$, $K_s = 0.658 \pm 0.065\ \mu M$, and $a = 63 \pm 7$.

accompanied by a protein conformational change that prevents reassembly of the tetramer.

The tetramer isolated on a preparative scale was shown to be deficient in flavin, containing only about 0.5 flavin per subunit. Purified dimeric 7-acetylflavo-GAPDH, however, contained 0.8 ± 0.1 flavin per binding site as determined from absorbance measurements and amino acid analysis. The latter is quite stable and can be stored at low temperature for several months without loss of activity. As discussed below, it is even stable enough to assay at 55 °C. In contrast, flavoenzyme **1b** derived from rabbit muscle enzyme[2] loses 30–40% of its activity overnight and is rapidly inactivated at elevated temperatures. It is possible that the instability of **1b** is due to a similar dissociation of the active tetramer into dimers. In this case, however, the dimeric structure may be very unstable and undergo further, irreversible, denaturation. Indeed, apo-GAPDH from rabbit muscle is significantly less stable than the holoenzyme.[22] It is possible that NAD^+, which has been shown to shift the dimer–tetramer equilibrium toward association,[23] protects the native enzyme precisely because it favors formation of the tetramer.

(21) One reviewer noted that multisubunit enzymes are frequently subject to cold inactivation, because the hydrophobic interactions that hold them together are less stable at 4 °C than at 25 °C. This fact suggests that the tetrameric flavoenzyme might undergo dissociation less readily at room temperature than at 4 °C. We do not have evidence that directly bears on this question, but note that the flavoenzyme derived from rabbit muscle, which is predominantly in the tetrameric state during our assays, is held together by a similar hydrophobic core. The muscle enzyme is definitely less stable at room temperature than at 4 °C. A full characterization of the bacterial tetramer is ongoing in our laboratory.

(22) Velick, S. F.; Hayes, J. E.; Harting, J. *J. Biol. Chem.* **1953**, *203*, 527–544.

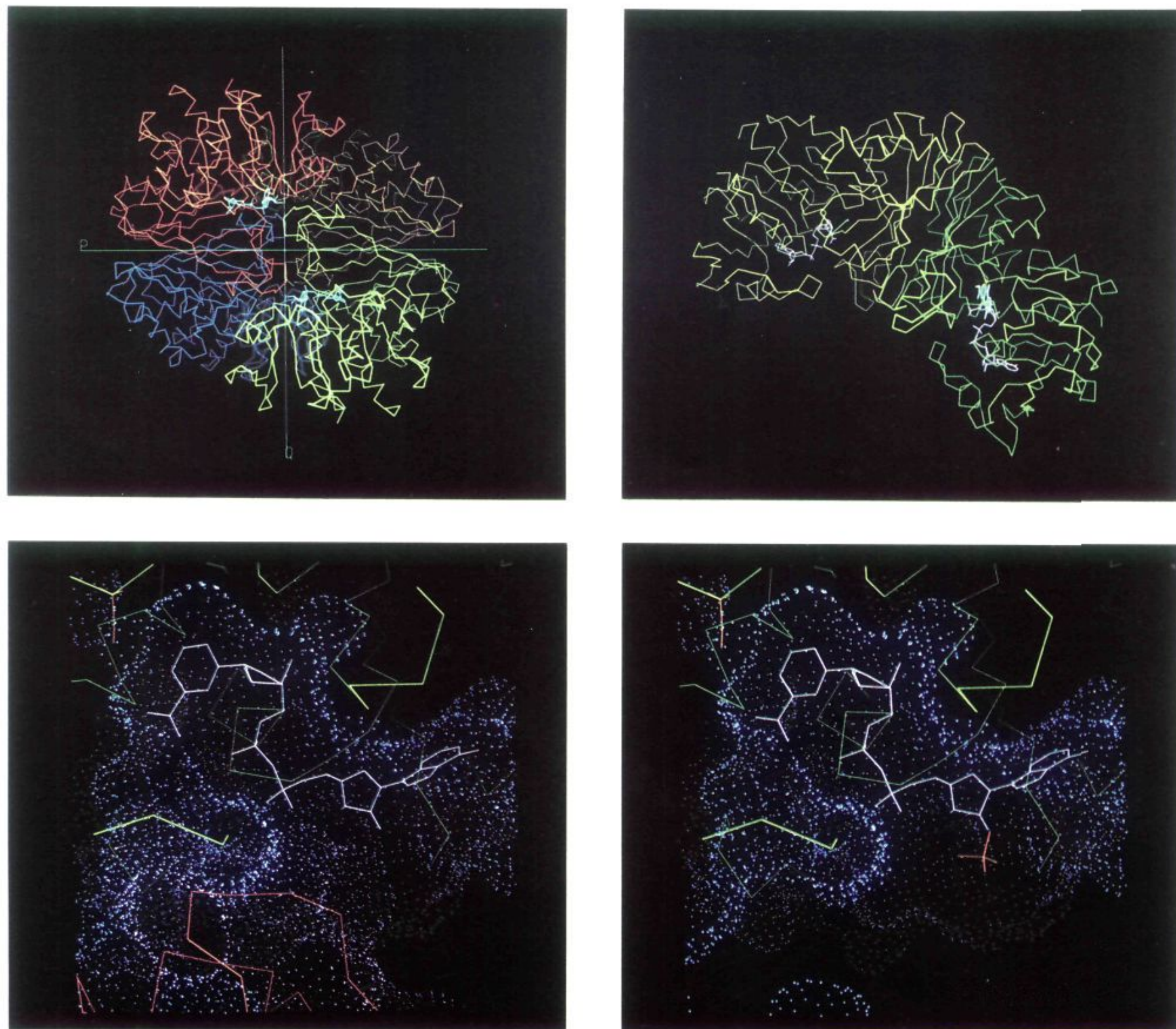


Figure 4. (a, top left) Trace of the peptide backbone of tetrameric holo-GAPDH from *B. stearothermophilus*. The four subunits are related by the diad axes shown (the *R* axis is perpendicular to the picture plane). Note the reciprocal intersubunit interactions across the *Q* axis for the green and red subunits. (b, bottom left) Close-up, with blue solvent accessible surface, of the NADH binding site of the green subunit of (a). The peptide loop from the red subunit that provides a hydrophobic floor to the adenosine binding site is shown with a magenta surface. The residues in the loop that are in closest contact with the adenosyl hydroxyl groups are Leu-187 and Pro-188. (c, top right) Proposed dimer model for 7-acetylflavo-GAPDH with bound NADH. The green-yellow (and red-blue) dimer is held together by close packing of the β-sheets of the respective monomers across the *P* axis (see (a) for the coordinate axes). (d, bottom right) Close-up of the NADH binding site of the green subunit in the proposed dimer. A phosphate moiety (red) has been added to the 2′-hydroxyl group of NADH, as in NADPH. Comparison with (b) shows that the steric and polar restrictions to NADPH binding would be relaxed following dissociation of the red subunit.

The increased stability of 7-acetylflavo-GAPDH from *B. stearothermophilus* may also be due to the reduced number of sulfhydryl groups per subunit relative to the rabbit muscle enzyme. It has only two thiols per subunit, one of which (Cys-149) is alkylated by the flavin analogue. Each subunit of the rabbit muscle enzyme, on the other hand, contains several cysteines in addition to Cys-149.[24] Thiol oxidation in the latter case, possibly catalyzed by the bound flavin moiety, may alter the protein conformation and result in the loss of activity.

Aerobic Oxidations of NADH by Bacterial 7-Acetylflavo-GAPDH 1c. We have studied the efficiency of the bacterial flavoenzyme by examining its ability to oxidize various 1,4-dihydronicotinamides. Formally, this reaction involves transfer of a hydride equivalent from the C-4 position of the dihydropyridine nucleus to the N-5 position of the isoalloxazine. In the presence of molecular oxygen reduced flavins like **2** are relatively rapidly reoxidized,[25] so that the process becomes catalytic in flavin. We predicted that NADH would be the optimal substrate for 7-acetylflavo-GAPDH, given the properties of the unmodified protein, and that it would be oxidized with *si*-face stereoselectivity. NADPH, which is not processed by the native enzyme, and hydrophobic *N*-alkyl-1,4-dihydronicotinamides were expected to be less good substrates. In each case the simple bimolecular reaction of 7-acetyl-10-methylisoalloxazine with the dihydronicotinamide served as the nonenzymatic model system against which the artificial flavoenzyme was judged.

Preliminary steady-state experiments with the crude bacterial semisynthetic enzyme (presumably a mixture of tetramer and dimer) and the column-purified dimer gave similar kinetic profiles with NADH. However, only the stable, purified dimer was characterized in detail. This species is an efficient catalyst for

(23) Hoagland, V. D.; Teller, D. C. *Biochemistry* **1969**, *8*, 594–602.

(24) (a) Perham, R. N. *Biochem. J.* **1969**, *111*, 17–21. (b) Harris, J. I.; Perham, R. N. *J. Mol. Biol.* **1965**, *13*, 876–884.

(25) Slama, J. T.; Radziejewski, C.; Oruganti, S. R.; Kaiser, E. T. *J. Am. Chem. Soc.* **1984**, *106*, 6778–6785.

Table I. Rate Parameters[a] for the Oxidation of Dihydronicotinamides by Bacterial 7-Acetylflavo-GAPDH

substrate	k_p, s^{-1}	$K_s \times 10^6$, M	a	k_p/K_s, M^{-1} s^{-1}
NADH	0.0647 ± 0.0017	0.853 ± 0.21	52 ± 11	75 900
[4-$^2H_2$]-NADH	0.0387 ± 0.0018	1.97 ± 0.27	14 ± 3.8	19 700
NADPH	0.0391 ± 0.0081	2.23 ± 0.35	4.0 ± 1.2	17 600
PNAH	0.0457 ± 0.0013	23.2 ± 2.40	1	1970
BNAH	0.0391 ± 0.0025	36.4 ± 7.50	1	1070

[a] Determined by fitting initial rate data, obtained at 340 nm in air-saturated buffer (50 mM Tris-HCl, pH 7.5, 25 °C), to eq 1 in the text.

the oxidation of NADH at 25 °C in air-saturated buffer, but the kinetic data could not be fitted to a hyperbolic Michaelis–Menten saturation curve. Instead, pronounced negative cooperativity was observed in plots of initial velocity vs substrate concentration (Hill coefficient, $n_H = 0.29 \pm 0.06$). Although it is not possible at this time to specify the precise kinetic mechanism that gives rise to the apparent cooperativity, one possible pathway is shown in Scheme I, where E_0E_0, S, and P represent the dimeric flavoenzyme, substrate, and product, respectively. This is a general sequential interaction model[26] in which binding of one substrate molecule to the dimeric enzyme increases the intrinsic dissociation constant for the adjacent site by a factor of a. The turnover number k_p for each binding site is assumed to be identical in this model, and the binding steps prior to the rate-determining step are assumed to be rapid and reversible. For catalysis to occur, the reduced enzyme-bound flavin that is formed after a single turnover must be reoxidized by molecular oxygen. This step is not shown explicitly in Scheme I but can take place in the presence or absence of bound NAD$^+$/NADH.

The general velocity equation for this model is given by eq 1.[26] The initial rate of reaction is given by v, and [S] and $[E]_T$ represent the substrate and total enzyme concentration, respectively. The

$$\frac{v}{[E]_T} = \frac{2k_p\left[\frac{[S]}{K_s} + \frac{[S]^2}{aK_s^2}\right]}{\left[1 + \frac{2[S]}{K_s} + \frac{[S]^2}{aK_s^2}\right]} \quad (1)$$

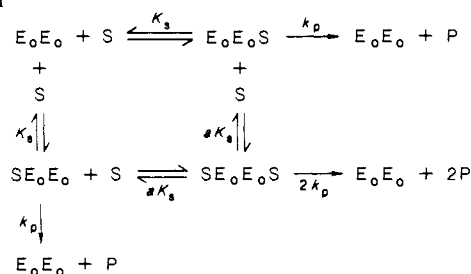
rate constants are defined as in Scheme I. The parameter a is an index for the degree of cooperativity in the system under the conditions of a given experiment. The larger the value of a, the greater the negative cooperativity. In the case of completely independent subunits, of course, $a = 1$. If flavin reoxidation is (partially) rate limiting, the kinetic parameters k_p and K_s will not be microscopic rate and equilibrium constants but will depend on oxygen concentration in a complex way.

The experimental data obtained for the oxidation of NADH by **1c** were fitted very well by eq 1 over a 1000-fold concentration range (Figure 3), and the kinetic parameters determined for this and other substrates are summarized in Table I. The tabulated experiments were performed in air-saturated buffer at 25 °C. Under these conditions, a is approximately 50 for NADH, indicating a significant degree of interaction between the subunits of the dimer. In addition, we have found that the values of k_p and K_s increase as the oxygen concentration in the medium is raised. In oxygen-saturated buffer, for example, the value of k_p for NADH oxidation is 1.7 times larger than that measured in air-saturated buffer. Clearly, reoxidation of the reduced flavin in the enzyme active site limits the overall turnover rate. Experiments with dideuteriated [4-$^2H_2$]NADH show, however, that the hydride-transfer step is also partially rate determining, since a 1.7-fold deuterium isotope effect is observed on k_p (Table I).

Although the oxidation of NADH by 7-acetylflavo-GAPDH is kinetically complex, comparison of the activity of this catalyst with that of an appropriate nonenzymatic model is essential for

(26) Segel, I. H. In *Enzyme Kinetics*; Wiley-Interscience: New York, 1975; pp 355–360.

Scheme I



evaluating the success of our design strategy. It is useful therefore to consider eq 1 in the limit of high and low substrate concentrations. When $[S] \gg aK_s$, for example, the velocity equation simplifies to the first-order rate expression (2). The turnover

$$v = 2k_p[E]_T = k_p[Fl]_T \quad (2)$$

number k_p for each flavin-containing subunit is thus analogous to the k_{cat} parameter from the more familiar Michaelis–Menten treatment of a noncooperative system. This pseudo-first-order rate constant is a measure of the efficiency of the chemical process at saturation. In a similar manner, as the concentration of S becomes very low ($[S] \ll K_s$), eq 1 reduces to the bimolecular rate expression (3). The apparent second-order rate constant

$$v = (2k_p/K_s)[E]_T[S] = (k_p/K_s)[Fl]_T[S] \quad (3)$$

k_p/K_s is analogous to the Michaelis–Menten k_{cat}/K_m parameter. It can be compared directly with the second-order rate constant for the nonenzymatic model reaction to obtain the maximum enzymatic rate acceleration at low substrate concentrations.

For the reaction of NADH with bacterial 7-acetylflavo-GAPDH, k_p/K_s is 75 600 M^{-1} s^{-1}. This corresponds to a 5800-fold rate acceleration over the process catalyzed by 7-acetyl-10-methylisoalloxazine in air-saturated buffer at low NADH concentrations ($k_2 = 12.9 \pm 0.3$ M^{-1} s^{-1}). These results compare favorably with an ca. 600-fold rate enhancement previously found[25] for 8-acetylflavopapain and its best substrate N-hexyl-1,4-dihydronicotinamide, suggesting the generality of the "chemical mutation" strategy for the design of protein-based catalysts. It is also interesting to compare the kinetic parameters obtained for bacterial 7-acetylflavo-GAPDH with those found[2] for the rabbit muscle enzyme **1b** under similar conditions. The muscle flavoenzyme does not exhibit cooperativity, and the kinetic data were shown[2] to conform to standard Michaelis–Menten behavior. The value of k_{cat}/K_m for oxidation of NADH by **1b** is about 70 times smaller than k_p/K_s for bacterial **1c**. Although the rate acceleration observed for the bacterial enzyme is significantly larger than that for the muscle enzyme, the difference is due primarily to the low value of K_s (0.825 μM) measured for the former as compared with the K_m value (25.4 μM) of the latter. The catalytic rate constant k_{cat} for oxidation of NADH by the muscle enzyme is only 2.4 times smaller than the k_p value determined for **1c**. In both cases, the absolute value of the latter parameters in air-saturated buffer is small. However, since k_p and k_{cat} are oxygen dependent, their magnitude does not reflect the true efficiency of the hydride-transfer step we have tried to model. In principle, the maximum possible turnover number could be extrapolated from steady-state experiments performed at several different oxygen concentrations. However, it is not possible to saturate the enzyme with experimentally accessible concentrations of oxygen. Therefore, as discussed below, we have employed rapid mixing techniques under anaerobic conditions to determine the microscopic rate constant for flavin reduction.

Thermal Stability and Stereospecificity of Bacterial 7-Acetylflavo-GAPDH 1c. As mentioned in the previous section, bacterial 7-acetylflavo-GAPDH is more robust than the semisynthetic enzyme from rabbit muscle. In order to characterize the thermal stability of the artificial catalyst further, we carried out the reaction of **1c** with NADH at elevated temperatures. The dimer was shown to be stable (i.e., >98% of all protein in a given sample) for the duration of the high-temperature assays by HPLC analysis. While the mesophilic enzyme is rapidly denatured when heated, the

Table II. Product Ratios for the Oxidation of Selectively Deuteriated NADH Derivatives by 7-Acetylflavo-GAPDH from *B. stearothermophilus* and Rabbit Muscle and by 7-Acetyl-10-methylisoalloxazine[a]

substrate	[4-²H]NAD⁺/[4-H]NAD⁺		
	B. stear.	muscle[2]	7-AcFl[2]
[4R-²H]NADH	3.35	13.3	1.86
[4S-²H]NADH	6.14	1.17	10.8

[a] The substrates were oxidized under the conditions described in Table I and the product ratios determined as described by Arnold et al.[12]

bacterial flavoprotein appears to function efficiently at temperatures as high as 55 °C. At the latter temperature in air-saturated buffer NADH is oxidized with a k_p value of 0.262 s^{-1}, a K_s value of 4.66 μM, and a k_p/K_s value of 56 200 M^{-1} s^{-1}. Under these conditions $a = 27$. The second-order rate constant for the nonenzymic model reaction is 23.1 M^{-1} s^{-1} at 55 °C, so the observed rate enhancement at low substrate concentrations is greater than 3 orders of magnitude. The turnover number k_p at 55 °C is almost 4 times larger than at 25 °C, while the second-order rate constant increases only 1.8-fold for the nonenzymatic model system. Although this result may reflect improved stabilization of the transition state for hydride transfer at high temperature, the complex dependence of the rate constants on oxygen concentration (which also changes with temperature) does not permit a definitive assessment of the situation.

Clearly, NADH is a good substrate for 7-acetylflavo-GAPDH from *B. stearothermophilus*. However, it is unlikely that the oxidation of NADH occurs with the nicotinamide ring in its normally bound position. The active site in each subunit of GAPDH is located between two large folding domains (Figure 2).[6] Introduction of the large isoalloxazine nucleus into the active site undoubtedly distorts the geometry of the binding pocket, possibly by acting as a wedge that can pry the domains apart. The ensuing conformational change could account for the shift of the dimer–tetramer equilibrium toward dissociation. In addition, the active sites in the resulting dimer are likely to be more open, which would allow the dihydropyridine portion of the substrate to rotate. Stereochemical studies of NADH oxidation with chirally deuteriated dihydronicotinamides support this suggestion. As shown in Table II, the oxidation of NADH by bacterial 7-acetylflavo-GAPDH is not very stereoselective. The product ratios[12] obtained for oxidation of [4A-³H]NADH and [4B-²H]NADH by **1c** do not show the expected preference for transfer of the *pro-S* hydrogen from the substrate. Rather, a modest preference for transfer of the *pro-R* hydrogen is evident, mirroring the *re*-face stereoselectivity seen[2] in the nonenzymatic oxidation of NADH by 7-acetyl-10-methylisoalloxazine. The results with the bacterial system contrast with our previous findings[2] for 7-acetylflavo-GAPDH from rabbit muscle, which catalyzes NADH oxidation with substantial *si*-face selectivity (Table II). The higher stereoselectivity in this case is consistent with the proposal that the rabbit muscle enzyme is active primarily as a tetramer. In the tetrameric flavoenzyme NADH is likely to be constrained to bind in a conformation close to that found in the unmodified protein.

Aerobic Oxidations of Other Dihydronicotinamides by Bacterial 7-Acetylflavo-GAPDH 1c. In order to assess the substrate specificity of thermophilic 7-acetylflavo-GAPDH, it was important to examine its ability to oxidize dihydronicotinamides other than NADH. The rate constants for the reaction of **1c** with NADPH, *N*-propyl-1,4-dihydronicotinamide (PNAH), and *N*-benzyl-1,4-dihydronicotinamide (BNAH) were therefore determined and are summarized in Table I.

In contrast to the reaction of NADH with **1c**, negative cooperativity is not observed in the oxidation of hydrophobic *N*-alkyl-1,4-dihydronicotinamides catalyzed by the bacterial flavoenzyme. Plots of initial velocity vs substrate concentration for both PNAH and BNAH were readily fit to Michaelis–Menten hyperbolic saturation curves, giving the tabulated k_p ($=k_{cat}$) and K_s ($=K_m$) values. Not surprisingly, neither compound is a particularly good substrate for the thermophilic enzyme. The rate accelerations, obtained by comparing k_{cat}/K_m with k_2 for the respective nonenzymic model, were only 2-fold for PNAH and 6-fold for BNAH. The selectivity of 7-acetylflavo-GAPDH for NADH contrasts with the substrate preferences found for the previous studied flavopapains[25] and presumably reflects the polar nature of GAPDH's active site. The fact that PNAH and BNAH do not elicit cooperative interactions argues against the possibility, not explicitly considered above, that the deviations from Michaelis–Menten behavior observed for NADH are actually due to the presence of two chemically distinct sites in the preparations of the enzyme dimer.

While the fact that PNAH and BNAH are not good substrates for bacterial 7-acetylflavo-GAPDH was not unexpected, the results obtained with NADPH were surprising. NADPH is not processed by the native enzyme,[6] but its oxidation is efficiently catalyzed by the semisynthetic flavoenzyme. The reaction of this compound with **1c** in air-saturated buffer, like that of NADH, is characterized by a negatively cooperative kinetic profile, although a is only about 4. Significantly, the value of K_s is only twice that found for NADH itself, and the value of the catalytic rate constant k_p is only about 1.7 times smaller than that observed for NADH. Consequently, k_p/K_s is 17 600 M^{-1} s^{-1}. Since the second-order rate constant for oxidation of NADPH by 7-acetyl-10-methylisoalloxazine is 22 M^{-1} s^{-1}, the effective enzymatic rate acceleration is about 900-fold for this substrate. We previously showed that NADPH was not a particularly good substrate for flavo-GAPDH from rabbit muscle. In that case, cooperativity was not observed and the data were fit to the Michaelis–Menten model to give $k_{cat} = 0.006\,13$ s^{-1}, $K_m = 1.08 \times 10^{-4}$ M, and $k_{cat}/K_m = 56.8$ M^{-1} s^{-1}. The rate acceleration for the muscle semisynthetic enzyme was therefore a mere 2.6-fold.

The observation that the bacterial flavoenzyme is dimeric provides a satisfying structural explanation for the ability of this catalyst to oxidize NADPH efficiently.[27] Examination of the 1.8-Å structural model of the native tetramer[4,5] (Figure 4a) reveals that there are important intersubunit contacts near the adenosine binding pocket. An irregular S-shaped peptide loop from the "red" subunit, for example, protrudes into the cleft in the "green" subunit that binds the adenosyl portion of NADH and provides a hydrophobic floor (Figure 4b). NADPH, which is phosphorylated at the 2′-hydroxyl group of the adenosyl ribose ring, cannot bind into this pocket because of severely unfavorable steric interactions with residues Leu-187 and Pro-188 in the intrusive loop of the adjacent subunit. However, were dimerization to occur along the crystallographic *Q* axis, the only true twofold axis in the protein, the unfavorable intersubunit contacts would be removed (Figure 4, parts c and d) permitting NADPH binding. The proposed dimeric species (Figure 4c) would be held together by the extensive interactions across the *P* axis that are also important in the native tetramer. Such contacts may be responsible for transmission of the negatively cooperative effects seen upon substrate binding, as well. We are currently trying to confirm the details of this proposal through crystallization and determination of the three-dimensional structure of the stable dimer. If rabbit muscle 7-acetylflavo-GAPDH were active primarily as a tetramer, the fact that it is a poor catalyst for the oxidation of NADPH could be explained by its inability to bind substrate effectively. The high K_m value observed in this case is consistent with this notion.[27]

Stopped-Flow Kinetic Studies of Bacterial 7-Acetylflavo-GAPDH 1c. In order to improve the general design of semisynthetic enzymes it is important to understand the origins of the catalytic properties of 7-acetylflavo-GAPDH. Insight into the workings of this semisynthetic enzyme can be gained by dissecting the steady-state rate parameters obtained under aerobic turnover conditions into microscopic rate constants. To accomplish this, we have studied the reduction of **1c** by NADH under anaerobic conditions.

(27) Preliminary experiments show that, when crude bacterial 7-acetylflavo-GAPDH is assayed immediately following its preparation (so that a significant amount of the tetrameric form is present in solution), the K_1 value for NADPH is roughly 10 times larger than that for NADH. This result contrasts with the mere 2-fold difference between the K_1 values for these two substrates obtained with the purified dimer and provides further supporting evidence for the postulated relationship between quaternary structure and substrate specificity in the various semisynthetic enzymes based on GAPDH.

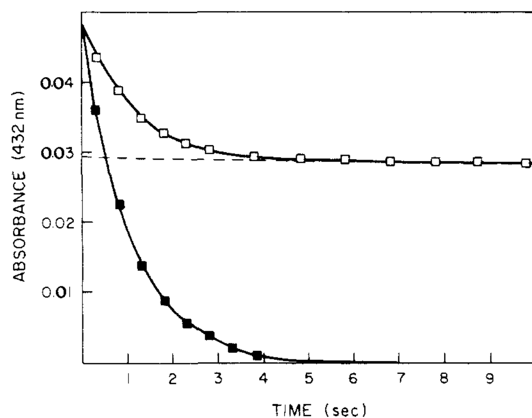


Figure 5. Typical transients observed at 432 nm for the reduction of dimeric 7-acetylflavo-GAPDH by excess NADH (□) and PNAH (■) under anaerobic conditions at 25 °C. Here, $[\text{7-acetylflavo-GAPDH}]_0$ = 1.37 μM, [NADH] = 1.09 mM, and [PNAH] = 0.826 mM. The biphasic time-course data obtained with NADH were fit to eq 4 (k_{fast} = 0.935 ± 0.030 s^{-1} and k_{slow} = (2.99 ± 0.9) × 10^{-3} s^{-1}); the dashed line indicates the separated slow step. A single first-order decrease in absorbance at 432 nm was found in the presence of PNAH (k_{obsd} = 0.929 ± 0.022 s^{-1}).

Using a conventional spectrophotometer, we observed burst kinetics in the disappearance of the flavin chromophore at 432 nm when **1c** and excess NADH were mixed in the absence of oxygen. With ca 0.1 mM NADH, a little less than half of the flavin molecules (42 ± 4%) were reduced instantaneously (<10 s), and the remainder at a much slower rate ($\tau_{1/2}$ = 1.91 ± 0.03 min). HPLC analysis of the flavoenzyme indicated that the preparation was about 95% pure, so the rapid reaction corresponds to reduction of half the active flavoprotein within experimental error. On the basis of documented[28] "half-of-the-sites" reactivity of unmodified GAPDH, we believe that the fast phase corresponds to reduction of only one flavin molecule per dimer. The second flavin in the enzyme is presumably reduced at a slower rate because of the much lower association constant for binding of a second substrate molecule. Half-of-the-sites reactivity is an extreme form of negative cooperativity and represents an important means of modulating activity typically unavailable to simple chemical catalysts.

To study the fast phase of the reaction we employed stopped-flow techniques. As shown in Figure 5 for a typical experiment (□), the fast and slow phases of 7-acetylflavo-GAPDH reduction by excess NADH could be cleanly resolved. The biphasic time-course data for individual transients were fit by computer to eq 4. The half-life of the slow phase was generally more than

$$A = \Delta A_{fast} \exp(-k_{fast}t) + \Delta A_{slow} \exp(-k_{slow}t) + A_{\infty} \quad (4)$$

2 orders of magnitude larger than that for the fast phase. Consequently, the total change in absorbance ΔA_0 corresponding to the reduction of all the flavins in the sample (i.e., $\Delta A_{fast} + \Delta A_{slow}$) could not be determined directly in the stopped-flow experiments. Instead, it was necessary to estimate the infinity value A_{∞} from the known concentration of the flavoenzyme; the estimate was confirmed by independent anaerobic reduction of the flavoenzyme by PNAH. In all cases examined the fast phase corresponded to reduction of roughly 40–50% of the flavin molecules as measured by $\Delta A_{fast}/\Delta A_0$. This ratio is subject to some error due to the uncertainty in ΔA_{slow}, but the calculated values are in good agreement with the stoichiometry of the burst determined by conventional spectroscopy. Qualitatively similar results were observed in the reaction of the enzyme with NADPH. The half-of-the-sites reactivity observed with NADH and NADPH, however, contrasts sharply with the results obtained when a hydrophobic *N*-alkyl-1,4-dihydronicotinamide was used as the reductant for 7-acetylflavo-GAPDH. As shown in Figure 5 (■), all of the flavin chromophore bleaches in a single pseudo-first-order process in the presence of excess PNAH. Thus, the anaerobic

(28) Levitzki, A. *J. Mol. Biol.* **1974**, *90*, 451–458.

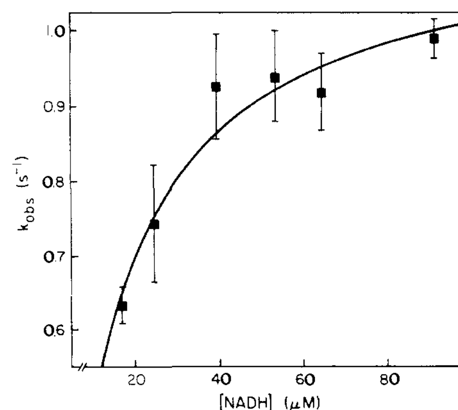
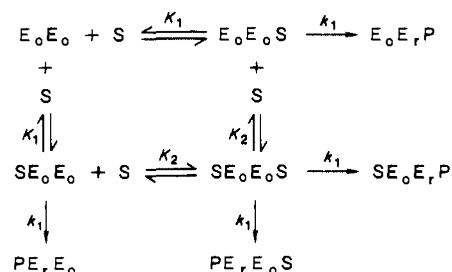


Figure 6. Concentration dependence of observed pseudo-first-order rate constant (k_{obsd} = k_{fast}) for reduction of dimeric 7-acetylflavo-GAPDH (1.37 μM) by NADH at pH 7.5 and 25.0 °C.

Scheme II



results are in qualitative agreement with findings from steady-state turnover experiments: NADH and NADPH elicit negatively cooperative kinetic profiles, while simple *N*-alkyl-1,4-dihydronicotinamides do not.

The fast-phase data obtained in the presence of excess NADH are consistent with Scheme II which depicts reduction of the oxidized enzyme dimer E_oE_o to the half-reduced form E_oE_r. The parameters k_1, K_1, and K_2 are microscopic rate and equilibrium constants and are related to k_{fast} by eq 5. A plot of k_{fast} vs NADH

$$k_{fast} = \frac{v}{[E]_T} = \frac{2k_1\left[\frac{[S]}{K_1} + \frac{[S]^2}{K_1K_2}\right]}{\left[1 + \frac{2[S]}{K_1} + \frac{[S]^2}{K_1K_2}\right]} \approx \frac{k_1[S]}{\left[\frac{K_1}{2} + [S]\right]} \quad (5)$$

concentration shows that saturation conditions can be approached (Figure 6). When $K_2 > [S] > K_1$, as in these experiments, eq 5 simplifies to give the standard Michaelis–Menten equation, with the effective dissociation constant for one subunit of the dimer, K_{S1}, equal to $K_1/2$. Fitting the data in Figure 6 to this simplified equation gave k_1 = 1.14 ± 0.05 s^{-1} and K_{S1} = 12.4 ± 2.4 μM. A double-reciprocal plot of the same data had a correlation coefficient of 0.98. The apparent bimolecular rate constant for the fast phase, k_1/K_{S1}, is therefore 91 900 M^{-1} s^{-1}. This value is very similar to the k_p/K_s value obtained under aerobic turnover conditions and is, moreover, of roughly the same magnitude as k_{cat}/K_m values observed for naturally occurring flavoenzymes.[1] The second-order rate constant for oxidation of NADH by 7-acetyl-10-methylisoalloxazine, on the other hand, is only 12.9 M^{-1} s^{-1} and is independent of oxygen concentration. Thus, the rate enhancement for 7-acetylflavo-GADPH at low NADH concentrations is greater than 7000-fold, which is 10-fold larger than that of our best case to date, the flavopapain-catalyzed oxidation of *N*-hexyl-1,4-dihydronicotinamide.[25]

It is interesting to compare the rate of reduction of half of the flavoenzyme by NADH near saturation with that by an equivalent concentration of PNAH. As shown in Figure 5, the half-lives of the two reactions are almost the same even though PNAH is about 75 times more easily oxidized by 7-acetyl-10-methylisoalloxazine than NADH.[2] Indeed, the efficiency of flavin reduction by

NADH, as measured by the pseudo-first-order rate constant k_1 (1.14 s^{-1}), is quite high. Naturally occurring flavoenzymes catalyze the oxidation of NADH with turnover numbers in the range 0.5–35 s^{-1}.[1] Given a suitable electron acceptor to reoxidize the flavin, the turnover number of bacterial 7-acetylflavo-GAPDH could therefore approach that of these biological systems. Unfortunately, electron-accepting dyes that were successfully employed with flavopapain,[29] dichloroindophenol and 3-(4',5'-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide, are less effective oxidants of reduced flavo-GAPDH than molecular oxygen. It is possible that binding of the bulky dye molecules at the active site is inhibited by bound product. On the other hand, preliminary experiments[30] with cytochrome *c* indicate that this molecule enhances enzyme turnover somewhat over that observed in air-saturated buffer, but it is still not the optimal oxidant. The search for better electron acceptors for reduced 7-acetylflavo-GAPDH therefore continues. Future efforts will also be directed toward improvement of the oxidative and reductive half-reactions by optimizing the electronic structure of the prosthetic flavin moiety and its site of attachment to the enzyme.

Conclusion. Bacterial GAPDH is an excellent template for the construction of stable semisynthetic flavoenzymes. The artificial enzyme we have examined, 7-acetylflavo-GAPDH, is a good catalyst for the oxidation of NADH and NADPH by molecular oxygen, showing rate accelerations at low substrate concentrations 3 orders of magnitude larger than the corresponding nonenzymatic model system. Moreover, the apparent bimolecular rate constant determined at low NADH concentrations is of enzymatic magnitude, as is the pseudo-first-order rate constant for hydride transfer in the enzyme–substrate complex determined by anaerobic stopped-flow procedures. The preference of 7-acetylflavo-GAPDH for hydrophilic substrates was predicted on the basis of a priori considerations of active site geometry and contrasts and complements the specificity for hydrophobic *N*-alkyl-1,4-dihydronicotinamides of the previously prepared flavopapains. Thus, by careful choice of protein template, the substrate specificity of our semisynthetic enzymes can be controlled in a systematic fashion.

Chemical mutation of existing tertiary structures promises to be a general strategy for the construction of artificial enzymes, especially if performed in conjunction with manipulations of the peptide backbone of the template protein. The availability of numerous natural variants makes GAPDH a particularly valuable model template. Our work with the bacterial enzyme has extended our earlier studies with rabbit muscle GAPDH. Using molecular graphics and crystallographic data, we have already been able to understand the differences in substrate specificity and stereoselectivity in the oxidation of dihydronicotinamides by the thermophilic and muscle 7-acetylflavo-GAPDHs in terms of their different quaternary structures. Examination of several other variants will permit a systematic investigation of the relationships between protein structure, catalysis, and regulation. Ultimately, site-directed mutagenesis using recombinant DNA techniques will enable us to construct even more sophisticated semisynthetic catalysts.

Acknowledgment. The partial support of this work by NIH Postdoctoral Fellowship AM-07232 (D.H.) and NSF Grant CHE-8218637 (E.T.K.) is gratefully acknowledged. We thank Desmond Roche for carrying out some preliminary experiments, Dr. Alan Wonacott for providing us with the coordinates of holo-GAPDH from *B. stearothermophilus*, and Drs. Arthur Olson and Alan Schwabacher for helpful discussions.

(29) Radziejewski, C.; Ballou, D. P.; Kaiser, E. T. *J. Am. Chem. Soc.* **1985**, *107*, 3352–3354.
(30) Kokubo, T., unpublished results.

Vibrational Circular Dichroism in the CH Stretching Region of (+)-3(*R*)-Methylcyclohexanone and Chiral Deuteriated Isotopomers

Teresa B. Freedman,* James Kallmerten, Elmer D. Lipp, Daryl A. Young, and Laurence A. Nafie*

Contribution from the Department of Chemistry, Syracuse University, Syracuse, New York 13244-1200. *Received April 13, 1987*

Abstract: The CH stretching Raman, FTIR, and vibrational circular dichroism (VCD) spectra of 3(*R*)-methylcyclohexanone and its chiral *2,2,6,6-d$_4$*, *4,4-d$_2$*, *5,5-d$_2$* and *methyl-d$_3$* isotopomers, and the Raman and FTIR spectra of racemic 3-methylcyclohexanone-*3-d$_1$* are presented and analyzed. Fourier self-deconvolution has been applied to the FTIR spectra to artificially enhance the resolution in order to determine individual band frequencies. Band assignments have been obtained on the basis of the spectral changes occurring due to selective deuteriation and from an analysis of the strong Fermi resonance interactions. The VCD spectral features of the five chiral isotopomers can all be understood in terms of the coupling of pairs of chirally oriented CH oscillators on adjacent carbon atoms and the removal of the degeneracy in the methyl modes.

The utility of vibrational circular dichroism (VCD)[1-4] as a probe of molecular conformation in solution arises in part from the fairly localized nature of many vibrational modes, which thus probe local geometry. Two general mechanisms for VCD intensity have been proposed that relate molecular conformation and the sign of the VCD signal due to localized vibration. The ring current mechanism[1,5,6] accounts for biased or monosignate VCD intensity due to local vibrational motion adjacent to or within a ring closed by covalent or intramolecular hydrogen bonding. Ring current enhancement has been observed for vibrational motion adjacent to

(1) Freedman, T. B.; Nafie, L. A. In *Topics in Stereochemistry*; Eliel, E. L., Wilen, S. H., Eds.; Wiley: New York, 1987; Vol. 17, pp 113–206.
(2) Keiderling, T. A. *Appl. Spectrosc. Rev.* **1981**, *17*, 189.
(3) Nafie, L. A. In *Advances in Infrared and Raman Spectroscopy*; Clark, R. J. M., Hester, R. E., Eds.; Wiley-Heyden: London, 1984; Vol. 11, p 49.
(4) Stephens, P. J.; Lowe, M. A. *Annu. Rev. Phys. Chem.* **1985**, *36*, 213.
(5) Nafie, L. A.; Freedman, T. B. *J. Phys. Chem.* **1986**, *90*, 763.
(6) Freedman, T. B.; Balukjian, G. A.; Nafie, L. A. *J. Am. Chem. Soc.* **1985**, *107*, 6213.

0002-7863/88/1510-0689$01.50/0 © 1988 American Chemical Society