Electron micrographs of DTT-polymerized **2** confirmed the presence of closed vesicles having a mean diameter of 635 ± 125 Å. In contrast to polymerized **1**, however, open-vesicle membranes were also observed. Whether or not these open structures are artifactual remains to be established.

Studies now in progress are focusing on a comparison of the membrane properties between DTT-polymerized disulfide vesicles derived from **1** and **2** and also their cross-linked analogues. Results of these studies will be reported in due course.

New Semisynthetic Flavoenzymes Based on a Tetrameric Protein Template, Glyceraldehyde-3-phosphate Dehydrogenase

Donald Hilvert and E. T. Kaiser*

Laboratory of Bioorganic Chemistry and Biochemistry The Rockefeller University, New York, New York 10021

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Recent reports from our laboratory have detailed a promising new strategy directed toward the rational design of artificial enzymes.[1,2] We have convalently modified the active site of papain with reactive analogues of flavin cofactors to yield chemically modified enzymes which are effective catalysts for the oxidation of *N*-alkyl-1,4-dihydronicotinamides. The most effective flavopapains exhibit substrate selectivity, show saturation kinetics, and rival the kinetic efficiency of naturally occurring flavoenzymes. The success of these first-generation semisynthetic enzymes demonstrates both the importance and potential of adapting binding sites of naturally occurring enzymes for the design of effective new catalysts active under mild aqueous conditions. We have now extended our studies to include a new protein template, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which allows for predictable substrate specificity and reaction stereospecificity from a priori considerations of active-site geometry.

GAPDH is a readily available enzyme which has been the subject of extensive physicochemical characterization.[3] The active enzyme is a tetramer composed of four identical subunits (M_r 36 000), each of which contains a discrete active site where both NAD^+/NADH and glyceraldehyde-3-phosphate (GAP) bind. X-ray diffraction studies[4] reveal that the nicotinamide cofactor binds within the pocket so that its pyridinium ring is near Cys-149. This sulfhydryl group, which is essential for catalysis, is believed to form a hemiacetal with GAP and can be selectively alkylated by a variety of reagents without destroying the NAD^+/NADH binding site. In this regard, the observation[5] of Rafter and Colowick that GAPDH promotes the reduction of enzyme-bound dichloroindophenol is particularly relevant.

We have utilized rabbit muscle GAPDH[6] for our studies to date and have carried out the alkylation of Cys-149 with 7α-(bromoacetyl)-10-methylisoalloxazine (**1**).[7] Because NAD^+ copurifies with GAPDH and is known to inhibit alkylation of the active-site thiol,[8] apoenzyme[9] (NAD^+ free, 1.5×10^{-5} M) was treated with a 25-fold excess of **1** in aqueous buffer for 1 h (50 mM pyrophosphate, pH 8.0, 25 °C). By use of this protocol, 0.9 ± 0.05 flavins are incorporated per subunit as estimated by anaerobic titration of the flavoprotein with dithionite[10] and Biuret protein determination.[11] The enzyme-bound flavin has λ_{max} 434 nm (ϵ 1.04×10^4 M^{-1} cm^{-1}); this is red-shifted by 7 nm relative to the λ_{max} of the analogous flavin free in solution.[7]

NADH is a good substrate for the flavo-GAPDH enzyme. Saturation kinetics were observed for the oxidation of this substrate in air-saturated buffer (pH 8.0, 25.0 °C, monitored at 340 nm), giving an apparent K_m value of 25.4 μM, while the product of the reaction (NAD^+) was determined to be a competitive inhibitor with a K_i value of roughly 330 μM. Under these conditions the apparent bimolecular rate constant $(k_{cat}/K_m)_{app}$ is 83 times larger than the second-order rate constant (k_2) for the oxidation of NADH promoted by the model compound 7-acetyl-10-methylisoalloxazine (**2**) (Table I). However, the observed rate acceleration depends on the concentration of oxygen in the medium, since both k_{cat} *and* k_{cat}/K_m increase with increasing amounts of oxygen, while k_2 for the model is oxygen independent. In oxygen-saturated buffer (25.0 °C), for example, the apparent bimolecular rate constant for the enzymatic reaction increases to 1550 M^{-1} s^{-1} which represents a 120-fold rate enhancement over the model system. This finding indicates that the catalytic efficiency of 7-acetylflavo-GAPDH, like that of flavopapain,[12] is limited by the flavin reoxidation step and, further, that flavin reoxidation can occur with substrate/product bound at the active site.[13] The turnover number is still small in oxygen-saturated buffer (0.0360 s^{-1}), although larger values of k_{cat} may be attainable if suitable artificial electron acceptors can be found.[12]

In contrast to the results obtained with NADH, significant rate accelerations were not observed for the oxidation of NADPH or simple *N*-alkyl-1,4-dihydronicotinamides (Table I). Although saturation kinetics were seen in the reactions promoted by the semisynthetic enzyme, the K_m values are greater than 150 μM for each of these substrates. Such substrate specificity (NADH > NADPH ~ BNAH ~ PNAH) was expected, given the known specificity of GAPDH itself,[3] and provides evidence that the flavin moiety is selectively incorporated into the active site of the protein.

Alkylation of GAPDH with the flavin derivative 8α-(bromoacetyl)-10-methylisoalloxazine (**3**)[1] yields an isomeric flavoprotein with properties very similar to those of 7-acetylflavo-GAPDH. For example, in air-saturated buffer the $(k_{cat}/K_m)_{app}$ value for NADH oxidation (475 M^{-1} s^{-1}) is 86-fold larger than the second-order rate constant for the model reaction in which 8-acetyl-10-methylisoalloxazine is the catalyst (5.5 M^{-1} s^{-1}). The differences in the rate constants for the oxidations of *N*-propyl- and *N*-benzyldihydronicotinamides catalyzed by the enzyme and the model systems are less than 2-fold. Flavopapains, on the other hand, exhibit a strong preference for the nonpolar *N*-alkyl-1,4-dihydronicotinamides as substrates over NADH,[1,7] and the specific enzymatic activity shows a greater dependence on the structure of the flavin analogue than is evident with the flavo-GAPDH's.[1,7]

We have also examined the stereochemistry of hydrogen transfer from chirally deuterated NADH molecules. The *pro-S* hydrogen of NADH is selectively transferred to diphosphoglycerate in the native protein,[3] and we predicted that a similar preference would be manifest in the reactions catalyzed by 7-acetylflavo-GAPDH. As the kinetic and product analysis data presented in Table II show, 7-acetylflavo-GAPDH does exhibit substantial *si*-face stereoselectivity. In contrast, transfer of the *pro-R* hydrogen is favored in the oxidation of NADH catalyzed by both the nonenzymatic model system[15] and flavopapain.[16] We are currently using stopped-flow techniques in order to determine the intrinsic

(1) Slama, J. T.; Radziejewski, C.; Oruganti, S. R.; Kaiser, E. T. *J. Am. Chem. Soc.* **1984**, *106*, 6778–6785.
(2) Kaiser, E. T.; Lawrence, D. S. *Science (Washington, D.C.)* **1984**, *226*, 505–511.
(3) Harris, J. I.; Waters, M. In "The Enzymes"; Boyer, P., Ed.; Academic Press: New York, 1976; Vol. XIII, Part C, pp 1–49.
(4) Moras, D.; Olsen, K. W.; Sabesan, M. N.; Buehner, M.; Ford, G. C.; Rossmann, M. G. *J. Biol. Chem.* **1975**, *250*, 9137–9162.
(5) Rafter, G. W.; Colowick, S. P. *Arch. Biochem. Biophys.* **1957**, *66*, 190–207.
(6) Boehringer-Mannheim Biochemicals.
(7) Levine, H. L.; Kaiser, E. T. *J. Am. Chem. Soc.* **1978**, *100*, 7670–7677.
(8) Trentham, D. R. *Biochem. J.* **1968**, *109*, 603–612.
(9) Havsteen, B. H. *Acta Chem. Scand.* **1965**, *19*, 1643–1651.
(10) Burleigh, B. D., Jr.; Foust, G. P.; Williams, C. H., Jr. *Anal. Biochem.* **1969**, *27*, 536–544.
(11) Cooper, T. G. "The Tools of Biochemistry"; Wiley-Interscience: New York, 1977; pp 51–53.
(12) Radziejewski, C.; Ballou, D. P.; Kaiser, E. T. *J. Am. Chem. Soc.* **1985**, *107*, 3352–3354.
(13) Massey, V.; Nishino, T. In "Flavins and Flavoproteins, Proceedings of the 6th International Symposium on Flavins and Flavoproteins"; Yagi, K., Yamano, T., Eds.; Japan Scientific Societies Press: Tokyo, 1980; pp 1–11.
(14) Arnold, L. J., Jr.; You, K.; Allison, W. S.; Kaplan, N. O. *Biochemistry* **1976**, *15*, 4844–4849.
(15) Upon reexamination, some of the data obtained earlier with **2**[16] were found to be incorrect. The more recently measured values, given in Table II, agree well with other published reports: San Pietro, A.; Kaplan, N. O.; Colowick, S. P. *J. Biol. Chem.* **1955**, *212*, 941–952.
(16) Levine, H. L.; Kaiser, E. T. *J. Am. Chem. Soc.* **1980**, *102*, 343–345.

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Table I. Rate Parameters[a] for the Oxidation of Dihydronicotinamides by 7-Acetylflavo-GAPDH and 7-Acetyl-10-methylisoalloxazine

substrate	7-AcFl-GAPDH, $(k_{cat}/K_m)_{app}$, M^{-1} s^{-1}	7-AcFl, k_2, M^{-1} s^{-1}	$(k_{cat}/K_m)_{app}/k_2$
NADH	1067 ± 31	12.9 ± 0.3	83
NADPH	57 ± 5	21.7 ± 1.4	2.6
PNAH[b]	624 ± 61	948 ± 14	0.65
BNAH[b]	487 ± 32	181 ± 7	2.7

[a] Determined at 340 nm in air-saturated buffer (50 mM EPPS, pH 8.0, 25 °C) by method of initial rates; ; [flavin] = 3.71×10^{-7} M; [superoxide dismutase] = 0.01 mg/mL; [catalase] = 0.086 mg/mL. [b] PNAH and BNAH refer to *N*-propyl- and *N*-benzyl-1,4-dihydronicotinamide, respectively.

Table II. Rate Parameters[a] and Product Ratios[14] for the Oxidation of NADH and Selectively Deuterated NADH Derivatives Catalyzed by 7-Acetylflavo-GAPDH and 7-Acetylflavin

substrate	7-AcFl-GAPDH $(k_{cat}/K_m)_{app}$, M^{-1} s^{-1}	7-AcFl-GAPDH (4-D)NAD$^+$/(4-H)NAD$^+$	7-AcFl k_2, M^{-1} s^{-1}	7-AcFl (4-D)NAD$^+$/(4-H)NAD$^+$
NADH	1067		13.4	
(4*R*-D)NADH	905	13.3	4.34	1.86
(4*S*-D)NADH	224	1.17	9.31	10.8
(4-D_2)NADH	113		2.02	

[a] Conditions as described in Table I.

preference for hydrogen transfer from the *si*-face of NADH in the enzymatic reaction and to elucidate the factors affecting the magnitude of the primary kinetic isotope effect.

In summary, we have developed a new series of semisynthetic flavoproteins based on GAPDH, a tetrameric protein template. In addition to rate accelerations of at least 120-fold for the oxidation of NADH, flavo-GAPDH's exhibit predictable substrate specificity and stereoselectivity opposite that of our earlier flavopapains. Our initial successes now make possible the preparation of more sophisticated artificial enzymes that take advantage of the tetrameric structure of the GAPDH template.

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Sterically Hindered Triplet Energy Transfer

J. C. Scaiano,*[1] W. J. Leigh,[2] M. A. Meador,[3] and P. J. Wagner*[3]

Division of Chemistry, National Research Council Ottawa, K1A 0R6 Canada
Chemistry Department, Michigan State University East Lansing, Michigan 48864

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For two decades now triplet energy transfer has played a key role in mechanistic investigations of photochemistry.[4] The usefulness of Stern–Volmer quenching studies in determining triplet lifetimes relies on accurate knowledge of energy-transfer rate constants. It is well established that *exothermic* bimolecular triplet energy transfer generally proceeds with rate constants that are close to diffusion controlled pretty much independent of donor and acceptor structure.[4] Several studies have found either small[5] or negligible[6] steric effects on this process. One of us has pointed out that energy transfer generally is so rapid at van der Waals separation of donor and acceptor molecules as to preclude significant steric effects.[6] We now wish to report some examples of significant steric effects on exothermic triplet energy transfer, examples that better define the geometric requirements of this process.

Table I. Representative Rate Constants for Quenching Ketones by Triplet Energy Transfer[a]

ketone	solvent	quencher	k_q, 10^9 M^{-1} s^{-1}
$PhCOCH_3$	benzene	1-MN[b]	8.4
xanthone	methanol	1-MN	9.3
p-MeOPhCOCH$_3$	benzene	dMH[c]	5.9
p-MeOPhCOCH$_3$	methanol	dMH	8.3
p-MeOPhCOt-Bu	benzene	dMH	5.0
p-MeOPhCOt-Bu	methanol	dMH	6.2
2-MeOPhCOPh	C_6H_5Cl	dMH	4.8
2-MeOPhCOPh	methanol	dMH	9.2
2-BzOPhCOPh	C_6H_5Cl	dMH	2.1
2-BzOPhCOPh	methanol	dMH	5.9
2,6-(MeO)$_2$PhCOPh	C_6H_5Cl	dMH	2.8
2,6-(MeO)$_2$PhCOPh	methanol	dMH	5.4
2,2′-(BzO)$_2$PhCOPh	C_6H_5Cl	dMH	0.8
phenanthrene	methanol	dMH	5.8
paracyclophane	benzene	dMH	2.8
$PhCOCH_3$	C_6H_5Cl	dMH	5.0
$PhCOCH_3$	C_6H_5Cl	COD[d]	2.0
KPC	methanol	dMH	2.3
KPC	C_6H_5Cl	dMH	0.88
KPC	methanol	COD	0.19
KPC	C_6H_5Cl	COD	0.08
xanthone	methanol	biphenyl	8.8
$PhCOC_3H_7$	benzene	biphenyl	2.5[e]
Ph_2CO	benzene	biphenyl	0.3[e]
KPC	benzene	biphenyl	0.0008

[a] Measured at room temperature; standard deviations typically ±5%. [b] 1-Methylnaphthalene. [c] 2,5-Dimethyl-2,4-hexadiene. [d] 1,3-Cyclooctadiene. [e] Reference 15.

We have measured rate constants for quenching of various triplet aryl ketones by conjugated dienes and some aromatics. The technique involves measurement of the triplet decay rate as a function of quencher concentration and in some cases verification by measuring sensitized triplet naphthalene absorption.[7] Table I summarizes the most pertinent results.

For a wide variety of phenyl ketones not substituted in either the ortho or α positions, rate constants at 27 °C cover the narrow ranges of $(6-8) \times 10^9$ M^{-1} s^{-1} in benzene and $(8-11) \times 10^9$ M^{-1} s^{-1} in methanol. Rate constants for quenching *p*-methoxypivalophenone[8] are 75% as large as for quenching *p*-methoxyacetophenone. This verifies our earlier conclusion that the effects of α-dimethyl substitution are minimal.[6] Likewise, 2,6-dimethoxy substitution results in only a halving of the rate constant.

Several *o*-benzyloxy ketones show larger effects. Thus each ortho benzyloxy group on benzophenone decreases k_q by a factor of 0.4. Since the rate constants for the ortho-substituted ketones are still partially diffusion controlled, we emphasize that the larger ortho groups indeed produce significant decreases in the rate for energy transfer between nearest-neighbor molecules. These decreases are disguised by the diffusion control.[6] For example, various workers have estimated the actual in-cage energy-transfer rate k_{et} as $(5-10) \times 10^{10}$ s^{-1}.[9,10] With a comparable rate for diffusion apart of donor and acceptor, observed rate constants in

(1) NRCC contribution No. 24676.
(2) Present address: Department of Chemistry, McMaster University, Hamilton, Ontario, Canada L8S 4M1.
(3) Michigan State University.
(4) Lamola, A. A. In "Techniques of Organic Chemistry"; Leermakers, P. A., Weissberger, A., Eds.; Wiley: New York, 1969; Vol. XIV.
(5) Herkstroeter, W. G.; Jones, L. B.; Hammond, G. S. *J. Am. Chem. Soc.* **1966**, *88*, 4777. Fry, A. J.; Liu, R. S. H.; Hammond, G. S. *Ibid.* **1966**, *88*, 4781.
(6) Wagner, P. J.; McGrath, J. M.; Zepp, R. G. *J. Am. Chem. Soc.* **1972**, *94*, 6883.
(7) Bays, J. P.; Encinas, M. V.; Small, R. D.; Scaiano, J. C. *J. Am. Chem. Soc.* **1980**, *102*, 727.
(8) Lewis, F. D.; Magyar, J. G. *J. Org. Chem.* **1972**, *37*, 2102. The methoxy group increases triplet lifetime and enhances triplet–triplet absorption.
(9) Anderson, R. W.; Hochstrasser, R. M.; Lutz, H.; Scott, G. W. *J. Chem. Phys.* **1974**, *61*, 2500.
(10) (a) Wagner, P. J.; Kochevar, I. *J. Am. Chem. Soc.* **1968**, *90*, 2232. (b) Saltiel, J.; Shannon, P. T.; Zafiriou, O. C.; Uriarte, A. K. *Ibid.* **1980**, *102*, 6799.

0002-7863/85/1507-5806$01.50/0