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Supplementary Material Available: Fractional coordinates, thermal parameters, bond distances, bond angles, and observed and calculated structure factors for vafzelin (**1**) and uvafzelin (**2**) (32 pages). Ordering information is given on any current masthead page.

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Chiral Discrimination in the Quenching of an Enzyme-Bound Fluorescent NAD^+ Analogue by an Optically Active Quencher

Sir:

The first stage in protein–ligand interaction involves a bimolecular association process. The experimentally observed rate constants for this reaction are in some cases diffusion controlled, one example being the interaction between superoxide dismutase and the superoxide ion whose rate constant was found to be 2.4×10^9 M^{-1} s^{-1}.[1] However, in most cases the associations between proteins and ligands are considerably slower, and the observed "on" rate constants for small ligands are typically between 10^7 and 10^8 M^{-1} s^{-1}.[2] These experimental values deviate largely from the results of calculations based on the Smoluchowski equation,[3] and models that use modifications of this equation have been developed in which the existence of a restricted target area on the enzyme surface is assumed.[4-7] A capture window model which also takes into account the size of the entering ligand was used by Szabo to correlate k_{on} values for the interaction between a series of ligands and hemoglobin with the ligand sizes.[5] Obviously the capture window model, which assumes a circular opening on the surface of the enzyme and no specific interactions with the ligand, is a crude approximation. Indeed, the rate constant obtained by using this model for the interaction between hemoglobin and O_2 was about tenfold larger than the experimental one, proving that the fit between ligand and protein is crucial for the efficiency of the association reaction. Thus the stereochemistry of a ligand, in addition to its size, may play a major role in the interaction.

In the present communication, we report what we believe to be the first direct observation of stereoselectivity in the interaction between an enzyme and a chiral molecule which is not a substrate and whose site of interaction is removed from the active site. Nevertheless this interaction may serve as a model for the first step of association between enzyme and ligand. The system studied was that of liver alcohol dehydrogenase (LADH) interacting with the two enantiomers of methionine, and the rate of association was determined from the quenching of fluorescence of a coenzyme analogue bound to the enzyme.

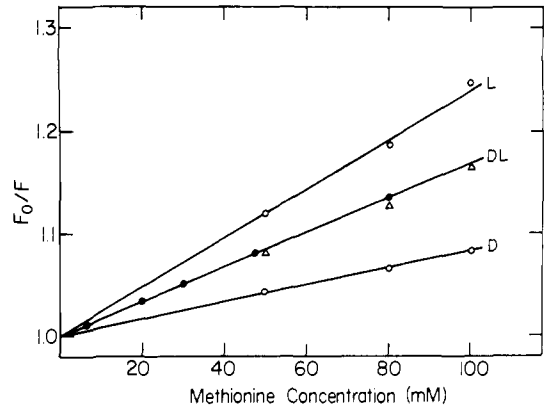

Figure 1. Stern–Volmer plots for quenching of fluorescence of the LADH–ϵNAD^+–pyrazole complex by L-, D-, and DL-methionine. The ternary complex was prepared in 0.05 M phosphate buffer (pH 7.3) and contained 1.2×10^{-5} M of both LADH and ϵNAD^+ and 1.7 mM pyrazole. (○) Quenching by L- or D-methionine. (●) Quenching by DL-methionine. (△) F_0/F values obtained by averaging the corresponding values of D- and L-methionine.

In a previous study we showed that the fluorescence of ϵNAD^+[8] bound to LADH is quenched both by iodide ions and by DL-methionine.[9] In the case of iodide the quenching was clearly dynamic, i.e., by collision between quencher and the fluorescent ethenoadenine ring, as evidenced by the excellent correspondence between the degree of quenching and the shortening of the fluorescence decay time.[9] Very similar behavior is observed when methionine is used as the quenching agent. The ratio between the fluorescence intensity of ϵNAD^+ bound to LADH in the absence of quencher and in the presence of 80 mM DL-methionine is 1.14. The fluorescence decay times of the coenzyme analogue in the same two samples were found to be 28.6 and 25.4 ns, respectively. Thus the shortening of the decay time (by a factor of 1.13) agrees well with the degree of fluorescence quenching, proving that the quenching by methionine is also solely dynamic. Since dynamic quenching occurs by direct contact between fluorophore and quencher, the latter must diffuse into the adenine binding site on LADH during the excited-state lifetime of the ϵNAD^+.

We found that while the rate constant for quenching of bound ϵNAD^+ by iodide was reduced only 1.6 times compared with that for the quenching of the free coenzyme analogue (i.e., from 3.9×10^9 to 2.5×10^9 M^{-1} s^{-1}), the ratio between the quenching rate constants of free and bound ϵNAD^+ by DL-methionine was above 12 (7.4×10^8 vs. 5.9×10^7 M^{-1} s^{-1}). Thus, while the "window" on the enzyme surface through which the ligands penetrate is large enough to allow fast diffusion of I^-, it considerably slows the diffusion of the bulky methionine. When the window and ligand are of comparable sizes, the rate of diffusion of the latter into the active site is determined to a large extent by thermal fluctuations in the protein which affect both the size and geometry of the window.[5] In such cases the rate of association between protein and ligand may depend not only on the size of the latter but also on its stereochemistry. Such chiral discrimination in the association is demonstrated in Figure 1 which presents Stern–Volmer plots for the quenching of the LADH–ϵNAD^+–pyrazole complex by D-, L-, and DL-methionine. The rate constants for fluorescence quenching of the ethenoadenine ring were calculated in each of the three cases by using the Stern–Volmer equation.[10] These constants were found to be $k_q^L = 8.4 \times 10^7$ M^{-1} s^{-1}; $k_q^D = 3.0 \times 10^7$ M^{-1} s^{-1}; and $k_q^{DL} = 5.9 \times 10^7$ M^{-1} s^{-1} (k_q^D, k_q^L, and k_q^{DL} being respectively the rate constants for quenching by D-, L-, and DL-methionine).

(1) E. M. Fielden, P. B. Roberts, R. C. Bray, D. J. Lowe, G. N. Mautner, G. Rotillio, and L. Calabrese, *Biochem. J.*, **139**, 49 (1974).
(2) G. G. Hammes and P. R. Schimmel, *Enzymes, 3rd, Ed.*, **11**, 67 (1970).
(3) M. W. Smoluchowski, *Z. Phys. Chem.*, *92*, 124 (1917).
(4) (a) T. L. Hill, *Proc. Natl. Acad. Sci., U. S. A. 72*, 4918 (1975); (b) **73**, 679 (1976).
(5) A. Szabo, *Proc. Natl. Acad. Sci., U. S. A.* **75**, 2108 (1978).
(6) H. Nakatani and H. B. Dunford, *J. Phys. Chem.*, **83**, 2662 (1979).
(7) P. Richter and M. Eigen, *Biophys. Chem.*, **2**, 255 (1974).

(8) Abbreviations used: ϵNAD^+, nicotinamide $1,N^6$-ethenoadenine dinucleotide; LADH, horse liver alcohol dehydrogenase.
(9) A. Gafni, *Biochemistry* **18**, 1540 (1979).
(10) In the Stern–Volmer equation: $F_0/F = 1 + k_q\tau_a[Q]$, F_0, F are the fluorescence intensities in absence and presence of quencher respectively, k_q is the quenching rate constant, τ_a is the fluorescence decay time in the absence of quencher (28.6 ns, see ref 9), and [Q] is the concentration of quencher.

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When several quenching agents which do not interact among themselves are present in solution, the Stern-Volmer equation will be

$$F_0/F = 1 + \sum_i k_q^i \tau_a [Q_i] \quad (1)$$

Since in the racemic mixture $[Q_L] = [Q_D] = {}^1/_2[Q_{DL}]$, it follows that for DL-methionine

$$\frac{F_0}{F} = 1 + (k_q^L + k_q^D)\tau_a \frac{[Q_{DL}]}{2} = 1 + \frac{(k_q^L + k_q^D)}{2}\tau_a[Q_{DL}]$$

Indeed, the observed rate constant for quenching by DL-methionine (5.9×10^7 M^{-1} s^{-1}) is close to the average of the rate constants for quenching by the pure antipodes (5.7×10^7 M^{-1} s^{-1}).

The marked stereoselectivity which exists in the interaction between LADH and methionine is revealed by the ratio of 3 between the rate constants for quenching. It is well established that the specificity of most enzymes toward the stereochemistry of their substrates is very high and in many cases even absolute, so that only one enantiomer participates in the enzymatic reaction.[11] It should be remembered, however, that this high stereospecificity is usually revealed in the interaction between an enzyme and its natural substrate, whose exact fit into the active site is crucial. Moreover, it is the overall reaction which shows the stereospecificity, while no indication is given as to the degree of discrimination between the two antipodes of the substrate in binding to the enzyme (many examples of ligands which bind rapidly and tightly but are inactive are known—i.e., inhibitors for enzymatic reactions; hence the inactivity of an antipode does not necessarily reflect lack of binding).

LADH is a somewhat unusual dehydrogenase in that it shows a broad specificity toward its substrates and oxidizes a wide range of primary and secondary alcohols.[12] This lack of sensitivity for the alkyl group of the alcohol being oxidized is thought to result from the fact that the alkyl binding domain in LADH is a hydrophobic site which combines with a variety of aliphatic groups with comparable affinities. This suggests that LADH may bind and react with both enantiomers of a secondary alcohol. Indeed it was found by Dickinson and Dalziel that LADH oxidizes both D- and L-2-butanol.[13] The oxidation rates of the two enantiomers differed by a factor of about 3, which is similar to the ratio of the rate constants for quenching by methionine reported here. This similarity is, most probably, a coincidence since the diffusion of methionine studied by us was into the adenine binding site of LADH and not into the substrate binding site. Also, as was mentioned above, the rate of enzymatic oxidation of an enantiomer does not necessarily reflect its rate of binding to the enzyme. Indeed, from the large rate constants for enzyme-ligand associations (10^7-10^8 M^{-1} s^{-1}), it is clear that this initial stage of the enzymatic reaction is not the rate-determining step in the oxidation and another process along the catalytic pathway must be responsible for the observed stereospecificity. The results of the present study clearly demonstrate chiral discrimination between two enantiomers of a small molecule interacting with LADH. Although methionine is not a substrate of this enzyme, the results obtained may be of a general significance in understanding the interactions in the first step of the enzymatic reaction—that of the primary association between enzyme and ligand.

(11) R. Bentley, "Molecular Asymmetry in Biology", Academic Press, New York, 1970.
(12) H. Sund and H. Theorell, *Enzymes, 2nd Ed.*, **7**, 25 (1963).
(13) F. M. Dickinson and K. Dalziel, *Nature (London)*, **214**, 31 (1967).

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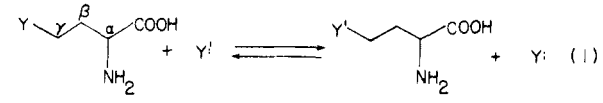
Stereochemical Course of γ Replacement on an Amino Acid Substrate in a Pyridoxal Phosphate Dependent Enzymatic Reaction

Sir:

Pyridoxal-P (PLP) is an obligate cofactor for a variety of enzyme-catalyzed transformations at the α, β, γ, or ω carbons of amino acid substrates by facilitated formation of stabilized substrate carbanion intermediates in catalysis. The subset of enzymes catalyzing γ replacement of a potentially good leaving group Y by an alternate nucleophile Y′ (eq 1), a formal substi-



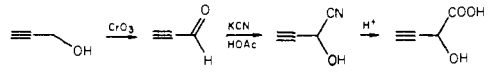
tution at an unactivated carbon, in fact acts via sequential formation of α and then β-substrate-carbanion PLP equivalents, followed by elimination of substituent Y to yield the key intermediate **2**.[1,2] This fully conjugated species is then captured by Y′ to yield a conjugate addition product which is unraveled by chiral α and β protonation to yield the product amino acid.

We report here for the first time the stereochemical outcome of such replacement, at the γ carbon of *O*-succinylhomoserine (**1**), during conversion to cystathionine (**3**), by the *Salmonella* enzyme, cystathionine γ-synthetase,[3,4] in the methionine biosynthetic pathway (Scheme I). We have recently demonstrated that L-vinylglycine (**4**), a natural compound,[5] is an excellent alternate substrate to succinylhomoserine, since **4** is converted to **2** merely by α-H abstraction from the amino acid-PLP aldimine complex.[6] Using specifically deuterated vinylglycines, we have determined first the stereochemistry of the second half-reaction, **2** → **3**, by degradation of cystathionine to homoserine. Then these [4-^{2}H]homoserines were chemically succinylated to **1a** or **1b** and converted enzymically to **3**, and thereby the stereochemistry of the first half-reaction, **1** → **2**, was uncovered.

(*Z*)-DL-[4-^{2}H]Vinylglycine (**4a**) was prepared by exchange of 2-hydroxy-3-butynoate[7] in $^2H_2O/O^2H^-$ (p^2H 9.5) followed by methylation to yield methyl 2-hydroxy-3-[4-^{2}H]butynoate. Partial reduction with hydrogen and Lindlar catalyst yielded *Z*-methyl [4-^{2}H]vinylglycolate. Deuterium enrichment at the 4*Z* position was estimated greater than 98.5% by ^{1}H NMR. This was then converted by standard steps[8] to (*Z*)-DL-[4-^{2}H]vinylglycine (**4a**)[9] (4% total yield from propynal). A parallel sequence of preparation, with omision of deuterium exchange of 2-hydroxy-3-butynoate but with use of deuterium gas in the reduction step, led to the product (*E*)-DL-[3,4-$^2H_2$]vinylglycine (**4b**).[9]

Incubation of **4a** or **4b** with purified[3,4] *Salmonella* cystathionine γ-synthetase and L-cysteine in 0.1 M potassium phosphate buffer (pH 8.4) at 37 °C produced monodeuteriocystathionine samples (102 and 67 mg, respectively), which precipitated out of solution

(1) Davis, L.; Metzler, D. *Enzymes, 3rd Ed.* **1972**, *17*, 42.
(2) Walsh, C. "Enzyme Reaction Mechanisms"; Freeman: San Francisco, CA; 1979.
(3) Guggenheim, S., Flavin, M. *J. Biol. Chem.* **1969**, *244*, 3722.
(4) Guggenheim, S., Flavin, M. *J. Biol. Chem.* **1971**, *246*, 3562 [102 mg (1 mmol) of (*Z*)-DL-[4-^{2}H]vinylglycine and 60 mg (0.5 mmol) of L-cysteine were dissolved in 5 mL of 50 mM potassium pyrophosphate buffer (pH 8.2); 5 units of cystathionine γ-synthetase (20 units/mg) added; incubated under argon atmosphere at 37 °C for 24 h].
(5) Dardenne, G.; Casimar, J.; Marlier, M. *Phytochemistry* **1974**, *13*, 1897.
(6) Johnston, M.; Marcotte, P.; Donovan, J.; Walsh, C. *Biochemistry*, **1979**, *18*, 1729.
(7)



(8) Rando, R. R.; *Biochemistry*, **1974**, *13*, 3859. Marcotte, P.; Walsh, C. *Ibid.* **1976**, *15*, 3070.
(9) (E)-DL-[3,4-^{2}H]Vinylglycine (**4b**): δ 5.42-5.48 (br, s 1 Sh), 4.23-4.25 (s, 1 H). (*Z*)-DL-[4-^{2}H]Vinylglycine (**4a**): δ 5.95 (m, 1 H), 5.53 (d, J = 11 Hz, 1 H), 4.22 (d, J = 7 Hz, 1 H).

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