creased fibrin deposition in flow separations adjacent to arterial bifurcations where low shear stresses are present and atherosclerotic plaques are often seen (*20*). More generally, enhancement of the fibrinolytic activity of the endothelial cell by shear stress may contribute to the nonthrombogenic function of the endothelial cell surface.

REFERENCES AND NOTES

1. C. F. Dewey, Jr., S. R. Bussolari, M. A. Gimbrone, Jr., P. F. Davies, *J. Biomech. Eng.* **103**, 177 (1981); R. M. Nerem, M. J. Levesque, J. F. Cornhill, *ibid.*, p. 172; G. E. White, M. A. Gimbrone, Jr., K. Fujiwara, *J. Cell Biol.* **97**, 416 (1983); A. Remuzzi, C. F. Dewey, Jr., P. F. Davies, M. A. Gimbrone, Jr., *Biorheology* **21**, 617 (1984); S. G. Eskin, C. L. Ives, L. V. McIntire, L. T. Navarro, *Microvasc. Res.* **28**, 87 (1984); M. J. Levesque and R. M. Nerem, *J. Biomech. Eng.* **107**, 341 (1985); C. L. Ives, S. G. Eskin, L. V. McIntire, *In Vitro Cell. Dev. Biol.* **22**, 500 (1986).
2. D. L. Fry, *Circ. Res.* **22**, 165 (1968); C. G. Caro, J. M. Fitz-Gerald, R. C. Schroter, *Proc. R. Soc. London Ser. B.* **117**, 109 (1971); H. L. Goldsmith and V. T. Turitto, *Thromb. Haemostasis* **55**, 415 (1986).
3. J. A. Frangos, S. G. Eskin, L. V. McIntire, C. L. Ives, *Science* **227**, 1477 (1985).
4. E. F. Grabowski, E. A. Jaffe, B. B. Weksler, *J. Lab. Clin. Med.* **105**, 36 (1985).
5. E. A. Sprague, B. L. Steinbach, R. M. Nerem, C. J. Schwartz, *Circulation* **76**, 648 (1987); P. F. Davies, C. F. Dewey, Jr., S. R. Bussolari, E. J. Gordan, M. A. Gimbrone, Jr., *J. Clin. Invest.* **73**, 1121 (1984); J. M. DeForrest and T. M. Hollis, *Am. J. Physiol.* **234**, 701 (1978); S. P. Olesen, D. E. Clapham, P. F. Davies, *Nature* **331**, 168 (1988).
6. D. C. Rijken, G. Wijngaards, J. Welbergen, *Thromb. Res.* **18**, 815 (1980); E. D. Sprengers and C. Kluft, *Blood* **69**, 381 (1987); J. A. Van Mourik, D. A. Lawrence, D. J. Loskutoff, *J. Biol. Chem.* **259**, 14914 (1984); E. G. Levin, *Blood* **67**, 1309 (1986).
7. D. Keber, M. Stegnar, I. Keber, B. Accetto, *Thromb. Haemostasis.* **41**, 745 (1979); T. K. Chan and V. Chan, *Thromb. Res.* **14**, 525 (1979); B. Wiman, G. Mellbring, M. Ranby, *Clin. Chim. Acta* **127**, 279 (1983); E. Angles-Cano *et al.*, *Thromb. Haemostasis* **58**, 843 (1987).
8. W. Speiser, E. Anders, K. T. Preissner, O. Wagner, G. Muller-Berghaus, *Blood* **69**, 964 (1987); D. C. Rijken, V. W. M. van Hinsbergh, E. H. C. Sens, *Thromb. Res.* **33**, 145 (1984); V. W. M. van Hinsbergh *et al.*, *Arteriosclerosis* **7**, 389 (1987); I. R. MacGregor and N. A. Booth, *Thromb. Haemostasis* **59**, 68 (1988).
9. Glass slides (75 by 35 mm; Fisher) were soaked in 0.5*M* NaOH for 2 hours, rinsed with deionized water, and autoclaved. Endothelial cells from six to eight umbilical cords (obtained within 6 hours of delivery) were pooled [see M. A. Gimbrone, Jr., *Prog. Hemostasis Thromb.* **3**, 1 (1976)] and seeded at about 3×10^5 to 6×10^5 cells per slide. Medium 199 (Gibco) with 20% heat-inactivated fetal calf serum (HyClone Laboratories), glutamine (0.30 mg/ml) (Gibco), penicillin and streptomycin (0.10 mg/ml each) (Gibco), and neomycin (0.20 mg/ml) (Gibco) was used for all experiments.
10. Constant fluid flow was produced by a hydrostatic pressure head in a recirculating system [see J. A. Frangos *et al.*, (*3*)]. Confluent monolayers were washed three times with phosphate-buffered saline (at 37°C) before each experiment. The cell cultures were mounted on parallel-plate flow chambers (monolayer surface area, 15 cm^2; channel width, 200 μm) and connected under sterile conditions to the flow systems each filled with 15 ml of medium. The systems were kept at 37°C with an air curtain incubator and at *p*H 7.4 by continuous gassing with a mixture of 5% CO_2 in air.
11. Total tPA (uncomplexed and inhibitor-bound) and uncomplexed PAI-1 (latent and active) were assayed with separate enzyme-linked immunosorbent assays [American Diagnostica: Imubind-5 tPA ELISA Kit, no. 122; Imubind PAI-1 ELISA Kit, no. 822]. The tPA kit was recalibrated (0 to 1500 pg/ml), yielding a highly linear calibration curve (typically, $r^2 > 0.99$) with a detection limit of 50 pg/ml. Each tPA medium sample (undiluted; 100-μl addition per well) was used as a blank against itself to eliminate nonspecific interference. We assayed the media samples (diluted 1:10 and 1:25) for PAI-1 following kit instructions without modification (by manufacturer's standard). Sample concentrations (calculated with 95% confidence limits) were used to calculate antigen production (per 10^6 cells).
12. Lactate dehydrogenase activity of conditioned medium or sonicated cell lysates was measured with Gilford Diagnostics reagent LD-P using a Gilford 2600 spectrophotometer. The LDH activity of fresh medium was subtracted from all samples. The percent lysis was calculated by dividing the sample activity by the activity of 10^6 lysed cells.
13. M. Hanss and D. Collen, *J. Lab. Clin. Med.* **109**, 97 (1987).
14. A. I. Schafer, M. A. Gimbrone, Jr., R. I. Handin, *Biochem. Biophys. Res. Commun.* **96**, 1640 (1980); J. S. Makarski, *In Vitro* **17**, 450 (1981).
15. T. Kooistra *et al.*, *Biochem. J.* **247**, 605 (1987).
16. A. J. Zonneveld *et al.*, *ibid.* **235**, 385 (1986); V. W. M. van Hinsbergh, E. D. Sprengers, T. Kooistra, *Thromb. Haemostasis* **57**, 148 (1987).
17. E. G. Levin, U. Marzec, J. Anderson, L. A. Harker, *J. Clin. Invest.* **74**, 1988 (1984).
18. J. J. Emeis and T. Kooistra, *J. Exp. Med.* **163**, 1260 (1986).
19. C. J. Jen and L. V. McIntire, *J. Lab. Clin. Med.* **103**, 115 (1984); J. L. Moake, N. A. Turner, N. A. Stathopoulos, L. Nolasco, J. D. Hellums, *Blood* **71**, 1366 (1988).
20. D. N. Ku, D. P. Giddens, C. K. Zarins, S. Glagov, *Arteriosclerosis* **5**, 293 (1985); D. Collen and I. Juhan-Vague, *Semin. Thromb. Hemostasis* **14**, 180 (1988).

21. Supported by NIH grants HL 18672 and HL 23016, grants NAS 9-17403 and NAG 9-207 from NASA, and grant C-938 from the Robert A. Welch Foundation. Special thanks to M. Estrella and L. Navarro for laboratory assistance.

20 September 1988; accepted 27 December 1988

Direct Brønsted Analysis of the Restoration of Activity to a Mutant Enzyme by Exogenous Amines

MICHAEL D. TONEY AND JACK F. KIRSCH*

A true Brønsted analysis of proton transfer in an enzyme mechanism is made possible by the chemical rescue of an inactive mutant of aspartate aminotransferase, where the endogenous general base, Lys^{258}, is replaced with Ala by site-directed mutagenesis. Catalytic activity is restored to this inactive mutant by exogenous amines. The eleven amines studied generate a Brønsted correlation with β of 0.4 for the transamination of cysteine sulfinate, when steric effects are included in the regression analysis. Localized mutagenesis thus allows the classical Brønsted analysis of transition-state structure to be applied to enzyme-catalyzed reactions.

ENZYMOLOGISTS HAVE BEEN ABLE TO adapt most of the tools of the physical organic chemist to the analysis of enzyme mechanisms. One of the most important of these, the linear free energy analysis of structure-reactivity correlations, is a powerful probe of transition-state structure. The principal structure-reactivity correlations are (i) those in which the reactant is varied and one obtains a linear free energy relation between the logarithm of the rate constant (log *k*) and a quantitatively defined structural parameter of the reactants (for example, the Hammett plot) and (ii) those in which the catalyst is systematically varied and the dependence of log *k* on the electronic nature of the catalyst helps to define the structure of the transition state. The classical example of the latter correlation is the Brønsted plot, in which log *k* is plotted against the pK_a of the general acid or general base catalyst. The slope of the resulting line is frequently interpreted as a measure of the degree of proton transfer between the reactant and catalyst, or of the amount of charge development, in the transition state.

Considerable experimental difficulties are encountered in attempting to apply linear free energy probes of mechanism to the study of enzyme-catalyzed reactions. The structural variations in the substrate required for Hammett analyses often result in incongruous interactions between the modified substrate and the well-defined active site. Nonetheless, substantial success has been achieved in many cases, particularly with relatively nonspecific enzymes. [See (*1*) for a review.] It has, however, not been previously possible to generate a Brønsted plot for a general acid or general base enzyme-catalyzed reaction, because the catalytic acid or base moiety is built into the enzyme structure (*2*). We report the application of site-directed mutagenesis to overcome this latter limitation and the development of a true Brønsted correlation for an enzyme-catalyzed reaction.

The ϵ-NH_2 moiety of Lys^{258} was postulated to be the base responsible for the proton transfer (*3*), which is central to the transamination mechanism (Fig. 1). This proposal receives support from the observation that *Escherichia coli* K258A [a mutant in which Lys^{258} has been replaced by Ala (*4*)] retains

Department of Biochemistry, University of California, Berkeley, CA 94720.

*To whom correspondence should be addressed.

less than 10^{-6} of the wild-type activity with Asp as substrate (*5*). K258A readily undergoes partial reactions with substrates: the pyridoxal-5′-phosphate (PLP) form combines with amino acids to produce external aldimines (**2**), whereas the pyridoxamine-5′-phosphate (PMP) form and α-ketoacids give ketimines (**4**), but further progress toward transamination is blocked for these intermediates (*6*). The x-ray crystal structure of K258A shows that the changes differentiating the mutant from wild-type enzyme are small and confined to the region immediately surrounding the site of the mutation (*7*). The mutant enzyme with Arg^{258} also lacks transaminase activity (*8*).

None of the other natural amino acids have functional groups similar to that of Lys. Thus the catalytic base in aspartate aminotransferase reactions cannot be systematically varied by site-directed mutagenesis. Given the 80 Å^3 difference in volume between Lys and Ala (*9*), the proposition that exogenous amines could partially replace the function of the Lys^{258} ε-NH_2 group was entertained. This proved to be the case. Shown in Fig. 2 is the dependence of the rate constant for the transamination of the external aldimine (**2**), formed from the PLP form of K258A and L-cysteine sulfinate, on 2-fluoroethylamine concentration. L-Cysteine sulfinate, an analog of the natural substrate L-aspartate (see Fig. 1), was used in these experiments because of its greater reactivity in the transamination reaction. The products of the reaction were equimolar quantities of pyruvate and PMP, which was expected because 3-sulfinylpyruvate, the transamination product of L-cysteine sulfinate, readily decomposes to pyruvate and bisulfite. Control experiments with PLP minus enzyme have shown that the nonenzymatic rate is insignificant at these concentrations.

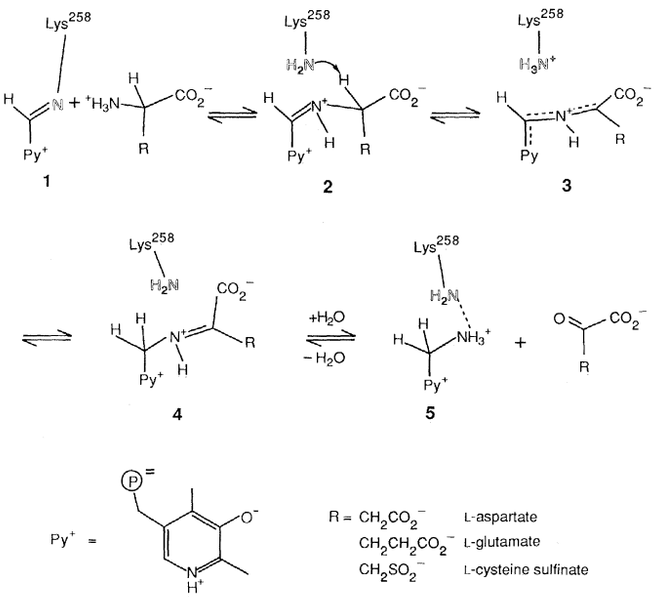

Fig. 1. Mechanism of amino acid transamination. The highlighted ε-NH_2 of Lys^{258} is the catalytic group that, in these experiments, is functionally replaced by amines added to the transamination reactions of K258A, a mutant aspartate aminotransferase in which Lys^{258} is changed to Ala. The identified structures are: **1**, internal aldimine; **2**, external aldimine; **3**, quinonoid; **4**, ketimine; and **5**, PMP (amino) enzyme. The catalytic transamination cycle is completed by the reverse of this sequence of steps with a second α-keto acid substrate.

Table 1. Rate constants, k_B, for the amine-assisted catalysis of L-cysteine sulfinate transamination by aspartate aminotransferase mutant K258A. The pK_a values were obtained as described in (*16*). The molecular volumes of the amines were calculated (*17–19*). We measured the time courses for the amino acid transamination half-reaction by monitoring the increase in absorbance at 330 nm due to the formation of enzyme-bound PMP. We calculated the k_{obs} values by fitting the absorbance data to a single exponential by nonlinear regression. Second-order rate constants were obtained by linear regression on k_{obs} versus [amine]. Conditions: 5 m*M* (saturating) L-cysteine sulfinate; 30 μ*M* K258A aspartate aminotransferase subunits; 100 m*M* CHES-tetramethylammonium hydroxide [CHES, 2-(*N*-cyclohexylamino)ethane sulfonic acid], *p*H 9.3; ionic strength maintained at 0.5 with tetramethylammonium chloride; 25°C. Amine stock solutions were titrated before use to *p*H 9.3 with tetramethylammonium hydroxide (*20*).

Amine	pK_a	Molecular volume (Å^3)	k_B* (M^{-1} s^{-1})
Methyl-	10.6	42.1	40 (2)
Ethyl-	10.6	60.9	1.30 (0.03)
Propyl-	10.5	79.8	0.27 (0.02)
Butyl-	10.6	98.7	0.009 (0.002)
Ethylenedi-	10.0	74.6	0.084 (0.002)†
Ethanol-	9.5	71.5	0.078 (0.003)
Ammonia	9.2	23.2	21 (2)
2-Fluoroethyl-	9.0	64.4	0.257 (0.009)
2-Cyanoethyl-	7.7	70.5	0.0108 (0.0003)
2,2,2-Trifluoroethyl-	5.7	71.6	0.0073 (0.0002)
Cyanomethyl-	5.3	51.1	0.050 (0.001)

* The k_B values are for the free base. Standard errors are given in parentheses. † k_B has been divided by 2 to correct for statistical effects. The neutral species is active.

The observed first-order rate constant, k_{obs}, is linearly dependent on the concentration of 2-fluoroethylamine at these concentrations. The *p*H dependence of the reaction (Fig. 2) demonstrates that the catalysis is due to the free base form of the amine, for example,

$$k_{obs} = \frac{k_B\,[\text{amine}]_{total}}{1 + ([H^+]/K_a)} + k_{solvent} \qquad (1)$$

The values of the rate constants, k_B, for catalysis of transamination by eleven primary amines are collected in Table 1. No evidence for saturation (at free base concentrations up to 250 m*M* in some cases) was observed, showing that amine binding is rather weak.

The primary amines listed in Table 1 vary in pK_a from 5.3 to 10.6. A simple Brønsted plot of log k_B versus pK_a of the amines shows a poor linear relation (slope $\beta = 0.3 \pm 0.2$), indicating that basicity alone does not adequately predict rate constants. Shown in Fig. 3 is a plot of log k_B versus solvent-excluded molecular volume for a subset of the amines, in which pK_a is effectively held constant. This plot shows a good linear relation [slope = (-0.062 ± 0.006) Å^{-3}] and indicates that a steric factor is important in determining rate constants for amine-assisted transamination. A similar exercise, plotting log k_B versus pK_a for the four amines of nearly constant molecular volume (70.5 to 74.6 Å^3, see Table 1), gave $\beta = 0.3 \pm 0.1$, isolating and emphasizing the importance of amine basicity.

The data were thus fitted to a multiple linear model (Eq. 2) with pK_a and solvent-excluded molecular volume as independent variables (*10*)

$$\log k_B = \beta(pK_a) + V\,(\text{molecular volume}) + c \qquad (2)$$

The values obtained from the least-squares fit are $\beta = 0.39 \pm 0.05$, $V = (-0.055 \pm 0.005)$ Å^{-3}, and c (the constant term) $= -0.7 \pm 0.5$. Conversely, when the data are fitted with the molecular volume as the sole independent variable, slope = (-0.05 ± 0.01) Å^{-3}. The value of V from the

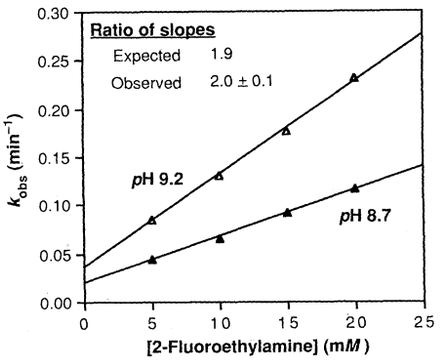

Fig. 2. The effect of 2-fluoroethylamine on the rate of the transamination reaction between *E. coli* K258A aspartate aminotransferase and L-cysteine sulfinate. The k_{obs} values were determined as described in Table 1. The expected ratio of the slopes of the lines is based on a pK_a of 9.0 for 2-fluoroethylamine with the free base as the active species.

multiple regression on the data for all eleven amines is the same (within error) as the value of the slope [(-0.062 ± 0.006) Å^{-3}] obtained for the line fit to the data in Fig. 3 (which includes only methyl-, ethyl-, propyl-, and butylamine), confirming the general nature of the effect of molecular volume on this reaction.

The total variation in the rate constants due to molecular volume is ten times that due to basicity for the set of amines investigated. Although the literature database for comparison is not extensive, the recent work of Estell *et al.* (*11*) on the binding of substrates to subtilisin mutants shows similar magnitudes for deleterious volume effects. Such large steric effects may indicate that, for reaction to occur, the added amine catalysts must be accommodated within the cavity created by the mutation from Lys^{258} to Ala, but attack from the solvent face of the aldimine (that is, from outside the cavity) is also possible.

A plot of log k_B − ($V \times$ molecular volume) versus pK_a is shown in Fig. 4. The volume correction reduces the plot of the data from three dimensions (Eq. 2) to two and permits presentation as a traditional Brønsted plot, graphically demonstrating the linear relation between log k_B and pK_a.

The value of k_{cat} for the transamination of L-cysteine sulfinate catalyzed by wild-type enzyme is 320 s^{-1} at pH 7 (*5*). The ϵ-NH$_2$ group of Lys^{258} thus has an effective molarity equivalent to 250M free base of ethylamine (320 s^{-1}/1.3M^{-1} s^{-1}; see Table 1), which has the same pK_a as free Lys and replaces most of the volume lost by substitution with Ala. The pK_a of ethylamine is 10.6; therefore, a total ethylamine concentration of 10^6M ($10^{3.6} \times 250M$) would be necessary to obtain this effective concentration of free base at pH 7. By maintaining the general base at the active site through covalent incorporation into the protein structure (that is, wild-type enzyme), high enzymatic efficiency is achieved.

The mechanistic interpretation of the β value of 0.4 is that the transition state has 40% of a full positive charge on the proton-accepting amine nitrogen, if we assume that the rate-determining step is Cα proton abstraction (Fig. 1). General base catalysis is kinetically indistinguishable from specific base–general acid catalysis (*12*), that is,

$$k[RNH_2][H_2O] = k\,K_a\,[RNH_3^+]\,[OH^-] \quad (3)$$

A mechanism involving specific base–general acid catalysis of transamination is one in which OH$^-$ abstracts the Cα proton from the amino acid to form the quinonoid (**3**) at equilibrium with the external aldimine (**2**). The rate-determining step in this mechanism is then the protonation of the quinonoid by RNH$_3^+$ acting as a general acid with Brønsted α = 0.6 (α is the slope in a plot of log k versus $-pK_a$ for general acid catalysis).

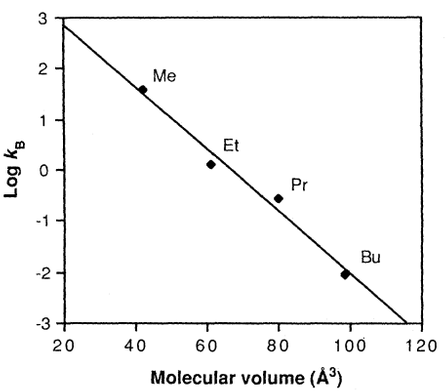

Fig. 3. Relation between log k_B and molecular volume for methyl- (Me), ethyl- (Et), propyl- (Pr), and butyl- (Bu) amines. The pK_a values for these four amines are virtually identical, so that only the molecular volume variations affect changes in the catalytic rate constant.

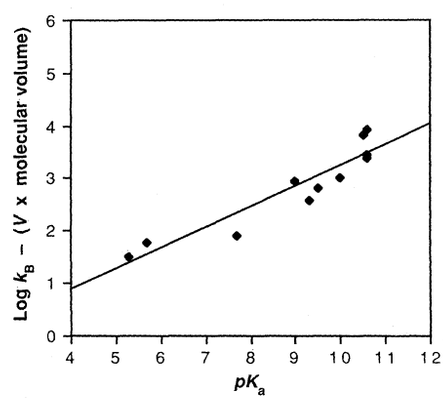

Fig. 4. Brønsted type plot for the acceleration of transamination between aspartate aminotransferase and cysteine sulfinate by exogenous amines. The slope of the line through the data is 0.4, the value of β for the reaction. We determined the values of V and β by linear regression on Eq. 2, using pK_a and molecular volume as independent variables.

Auld and Bruice (*13*) have studied general base catalysis of the nonenzymatic transamination reaction between Ala and 3-hydroxypyridine-4-aldehyde, an analog of PLP. They found β values ranging from 0.24 to 0.64 for the different protonic forms of the aldimine complex. The β of 0.4 measured for the enzymatic reaction with cysteine sulfinate falls within this range, but further comparison is not warranted because of the difference in amino acids.

The acceleration of enzymatic reactions by added proton transfer catalysts is not without precedent. Silverman and Tu showed that the carbonic anhydrase reaction is accelerated by low concentrations of added buffers and proposed that the buffer assists in a rate-limiting proton transfer step (*14*). More recently, Carter and Wells demonstrated that the role of the His in the catalytic triad of a serine protease could be functionally replaced in part (after substitution by Ala by site-directed mutagenesis) by a His contained within the peptide substrate (*15*). In this latter case, the mutant enzyme has a greatly enhanced specificity toward protein sequences containing His. In the aspartate aminotransferase experiments a deleted catalytic group has been functionally replaced by various added small molecules, allowing systematic variation of the catalytic properties and an analysis of transition-state structure. Functional replacement of catalytic groups at enzyme active sites by small molecules may prove to be widely applicable to inactive mutant enzymes in which amino acids with small side chains have replaced catalytic residues.

REFERENCES AND NOTES

1. J. F. Kirsch, in *Advances in Linear Free Energy Relationships*, N. B. Chapman and J. Shorter, Eds. (Plenum, New York, 1972), chap. 8.
2. The plot under consideration here is of the classical Brønsted type, in which the pK_a of the catalyst, an independently determined parameter, is plotted against the logarithm of the rate constant for the catalyzed reaction. It is differentiated from the relations recently put forth by A. R. Fersht, R. J. Leatherbarrow, and T. N. C. Wells [*Nature* **322**, 284 (1986); *Biochemistry* **26**, 6030 (1987)] and by S. J. Benkovic, C. A. Fierke, and A. M. Naylor [*Science* **239**, 1105 (1988)], in which the effects of varying the enzyme structure by site-directed mutagenesis are to perturb simultaneously two or more variables of the same reaction, for example, a rate and an equilibrium constant. Some of these separate perturbations have been observed to be linearly correlated with each other. The variables in some of these analyses are not necessarily independent [D. A. Estell, *Protein Eng.* **1**, 441 (1987)], and thus their validity is questionable. These latter relations might be termed "internal" versus the "external" Brønsted or Hammett class.
3. J. F. Kirsch *et al.*, *J. Mol. Biol.* **174**, 497 (1984); *Transaminases*, P. Christen and D. Metzler, Eds. (Wiley, New York, 1985), chap. 5.

4. B. A. Malcolm and J. F. Kirsch, *Biochem. Biophys. Res. Commun.* **132**, 915 (1985).
5. M. D. Toney and J. F. Kirsch, unpublished results.
6. J. F. Kirsch *et al.*, in *Biochemistry of Vitamin B_6*, T. Korpela and P. Christen, Eds. (Birkhauser Verlag, Basel, 1987), pp. 59–67.
7. D. L. Smith, D. Ringe, W. L. Finlayson, J. F. Kirsch, *J. Mol. Biol.* **191**, 301 (1986); D. L. Smith, S. C. Almo, D. Ringe, M. D. Toney, unpublished results.
8. S. Kuramitsu *et al.*, *Biochem. Biophys. Res. Commun.* **146**, 416 (1987); H. Kagamiyama, personal communication.
9. C. Chothia, *Nature* **254**, 304 (1975).
10. Substitution of solvent-accessible surface area for solvent-excluded molecular volume in Eq. 2 gave a much poorer fit to the data.
11. D. A. Estell *et al.*, *Science* **233**, 659 (1986).
12. W. P. Jencks, *Catalysis in Chemistry and Enzymology* (McGraw-Hill, New York, 1969), pp. 182–199.
13. D. S. Auld and T. C. Bruice, *J. Am. Chem. Soc.* **89**, 2098 (1967).
14. D. N. Silverman and C. K. Tu, *ibid.* **97**, 2263 (1975).
15. P. Carter and J. A. Wells, *Science* **237**, 394 (1987).
16. The pK_a values were taken from the *Handbook of Biochemistry and Molecular Biology*, H. A. Sober, Ed. (Chemical Rubber Company, Cleveland, 1968), pp. 150–189, except for that of 2-fluoroethylamine, which we estimated by extrapolation, using the pK_a values of ethylamine, 2,2-difluoroethylamine, and 2,2,2-trifluoroethylamine.
17. We calculated molecular volumes by first building and energy-minimizing the molecules, using the graphics program MacroModel (*18*). The coordinates obtained were used in the programs of Connolly (*19*), with a probe radius of 1.4 Å. The atomic radii used were as follows: C, 1.7 Å; H, 1.2 Å; N, 1.5 Å; F, 1.35 Å; O, 1.4 Å; triple-bonded C, 1.3 Å; triple-bonded N, 1.3 Å.
18. W. C. Still, MacroModel 1.5 (Chemistry Department, Columbia University).
19. M. L. Connolly, *J. Am. Chem. Soc.* **107**, 1118 (1985).
20. CHES was purchased from Sigma. Amine hydrochlorides were purchased from Aldrich and were the highest grades available. Ethanolamine · HCl and ethylenediamine · 2HCl were recrystallized before use. K258A was prepared essentially as described for the wild-type enzyme (*7*).
21. We thank S. Holbrook for help in calculating the molecular volumes used herein. Supported by NIH grant GM35393. M.D.T. was supported in part by NIH training grant GM07232.

26 August 1988; accepted 21 November 1988

Amyloid β Protein Enhances the Survival of Hippocampal Neurons in Vitro

JANET S. WHITSON, DENNIS J. SELKOE, CARL W. COTMAN*

The β-amyloid protein is progressively deposited in Alzheimer's disease as vascular amyloid and as the amyloid cores of neuritic plaques. Contrary to its metabolically inert appearance, this peptide may have biological activity. To evaluate this possibility, a peptide ligand homologous to the first 28 residues of the β-amyloid protein (β1-28) was tested in cultures of hippocampal pyramidal neurons for neurotrophic or neurotoxic effects. The β1-28 appeared to have neurotrophic activity because it enhanced neuronal survival under the culture conditions examined. This finding may help elucidate the sequence of events leading to plaque formation and neuronal damage in Alzheimer's disease.

IN ALZHEIMER'S DISEASE (AD), INSOLUBLE fibrils of β-amyloid (A4) protein accumulate in cerebral blood vessels (*1*) and in neuritic plaques (*2, 3*). Within the plaques, found in various regions of the cerebral cortex and in the hippocampus (*4, 5*), a central core containing primarily β-amyloid is surrounded by a spherical cluster of dystrophic neurites and glial cells (*5*). Although the brains of normal elderly adults exhibit neuritic plaques with their associated amyloid deposits, the density of the lesions is much greater in AD and, indeed, is a pathological marker by which diagnosis is confirmed (*5*). Thus, β-amyloid deposition could be thought of as a primary event in the pathological cascade of AD. It is also possible, however, that β-amyloid itself may constitute part of a reactive, albeit aberrant, plasticity response to the neuronal loss that accompanies the disorder. The possibility that the AD brain is capable of compensatory responses has been suggested from studies in which rats given lesions of the entorhinal cortex (to mimic the pathology observed in this region in AD) exhibited sprouting of neurites positive for acetylcholinesterase (AChE) in the outer molecular layer of the dentate gyrus. Detailed characterization of human hippocampal pathology in AD has revealed a similar sprouting response and has shown that AChE-positive neuritic plaques also accumulate in that dentate layer (*6*). Thus, Geddes and others have indicated the possible preferential location of neuritic plaques in regions of active sprouting and have proposed that an initial stage of neuritic plaque formation may be an aberrant sprouting response caused by local injury-induced increases in neurotrophic activities (*6*). As the direct effects of β-amyloid protein on brain cells have not been determined, questions of the trophic or toxic properties of β-amyloid remain unresolved.

In the present study we examined the effect of a synthetic β-amyloid peptide (β1-28) on cell survival and neuritic outgrowth in primary cultures of hippocampal neurons. The β1-28 peptide (*7*) consists of the first 28 residues of the β-amyloid protein (estimated to be 40 to 42 residues in all) and is thought to represent a portion of the extracellular domain of the amyloid precursor (*8, 9*).

In initial experiments, β1-28 was added to the medium in which embryonic rat hippocampal cells were plated and grown for 2 days. In these low-density cultures, the numbers of surviving cells was quantitated on day 2 as a percentage of the number of live cells counted 1 hour after plating. Cultures were treated with β1-28 (0, 10, and 100 μg/ml) in four separate preparations and triplicate wells were used for each concentration (Fig. 1). In the absence of β-amyloid peptide, neuronal survival at the end of 2 days was only 36% (35.6 ± 6.4). When β1-28 was included in the medium at 10 and 100 μg/ml, these values increased to 60% and 90%, respectively (60.2 ± 9.6, 90.1 ± 12.2: $P = 0.0016$, $n = 12$ ± SEM). To assess the time course of this survival effect we prepared cultures and determined cell survival after 1, 3, 5, or 9 days (Fig. 2). The percentage of neurons sustained by β1-28 at day 1 was nearly 100% (compared to about 25% without β1-28), but dropped sharply between days 3 and 5. By days 7 and

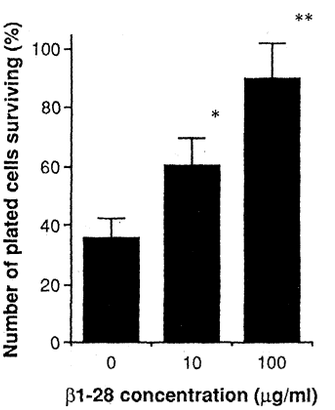

Fig. 1. Survival of hippocampal neurons as a function of β1-28 concentration. Hippocampal cells from embryonic day 18 Sprague-Dawley rats were plated in polylysine-coated 24-well culture plates (*10*). β1-28 was included in Brewer's B18 serum-free culture media (*10*) when cells were plated. After 2 days, cells were counted and survival calculated as a percentage of day 0 plating counts [$^{**}P < 0.01$, $^{*}P < 0.09$, Fisher probable least-squares difference (PLSD)]. Values are mean ± SEM; $n = 12$. Analysis of variance (ANOVA): $F(2,35) = 7.854$, $P < 0.002$.

J. S. Whitson and C. W. Cotman, Department of Psychobiology, University of California at Irvine, Irvine, CA 92717.
D. J. Selkoe, Department of Neurology (Neuroscience), Harvard Medical School, Boston, MA 02115 and Center for Neurologic Diseases, Brigham and Women's Hospital, Boston, MA 02115.

*To whom correspondence should be addressed.