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A Kinetic Analysis of the Enhanced Catalytic Efficiency of Papain Modified by 2-Hydroxy-5-nitrobenzylation[†]

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ABSTRACT: The incorporation of a 2-hydroxy-5-nitrobenzyl (HNB) group into the covalent structure of papain, through reaction of the enzyme with 2-chloromethyl-4-nitrophenyl (*N*-carbobenzoxy)glycinate, resulted in altered kinetics of hydrolysis of benzoyl-L-arginyl substrates. Comparison of k_{cat}/K_m ratios at pH 6.5 revealed that hydroxynitrobenzylation of papain led to a 24% increase in activity toward benzoyl-L-arginine ethyl ester (BzArgOEt) and a 240% increase in activity toward benzoyl-L-arginine *p*-nitroanilide (BzArgNan). These changes in activity reflected a decrease in the apparent Michaelis constants for both substrates, coupled with a 28% decrease and a 27% increase in k_{cat} values for BzArgOEt and BzArgNan, respectively. Analysis of the pH-dependent steady-state kinetics of BzArgNan hydrolysis revealed that hydroxynitrobenzylation had resulted in a shift in pK_a values of papain's functional groups from 4.3 and 8.2 to 4.6 and 7.7, respectively. The increased efficiency of BzArgNan hydrolysis could be attributed to a sevenfold increase in k_2(lim), the rate constant governing the enzyme "acylation" step. By contrast, no significant change in the rate constant governing "deacylation" resulted from hydroxynitrobenzylation of papain.

In their X-ray crystallographic analyses of papain (EC 3.4.4.10), Drenth *et al.* (1968, 1970, 1971) discovered the presence of several tryptophyl residues in the immediate vicinity of the active site of the enzyme. In particular, the indole side chain of Trp-177 is located at the surface of the enzyme molecule next to the imidazole ring of His-159 and at a distance of about 6 Å from the sulfur atom of Cys-25 in the crystalline state. Involvement of tryptophyl residues in the catalytic activity of papain has been implicated through a variety of chemical modifications, including proflavine-sensitized photooxidation (Jori and Galiazzo, 1971), oxidation with *N*-bromosuccinimide (Kirschenbaum, 1971), and reaction of papain with sodium bisulfite, tetranitromethane, and 2-hydroxy-5-nitrobenzyl bromide (Morihara and Nagami, 1969). In addition, the fluorescence of papain has been

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[†] From the Department of Biochemistry, North Carolina State University, Raleigh, North Carolina 27607. *Received August 27, 1973.* This investigation has been supported in part by National Science Foundation Research Grant GB-33472, and is a contribution from the Schools of Agriculture and Life Sciences and Physical and Mathematical Sciences. Paper No. 4044 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, N. C.

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TABLE I: Kinetics of Papain- and HNB-Papain-Catalyzed Reactions with BzArgOEt and BzArgNan at pH 6.5.

Papain	Substrate	K_m(app) × 10^3 M	k_{cat} (sec^{-1})	k_{cat}/K_m(app) × 10^{-3} (M^{-1} sec^{-1})
Native	BzArgOEt	16.9 ± 1.9	26.90 ± 1.80	1.59
HNB	BzArgOEt	9.8 ± 0.6	19.30 ± 0.60	1.97
Native	BzArgNan	2.95 ± 0.16	0.73 ± 0.03	0.25
HNB	BzArgNan	1.09 ± 0.10	0.93 ± 0.04	0.85

TABLE II: Kinetics of HNB-Papain-Catalyzed Hydrolysis of Benzoyl-L-arginine *p*-Nitroanilide.[a]

pH	K_m(app) (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m(app) (M^{-1} sec^{-1})	k_2 (sec^{-1})	k_3 (sec^{-1})
3.94	2.44 ± 0.34	0.38 ± 0.03	154.1 ± 34.5	22.9	0.45
4.48	1.86 ± 0.17	0.73 ± 0.04	390.0 ± 57.7	5.82	0.83
4.94	1.39 ± 0.12	0.91 ± 0.04	651.1 ± 87.8	9.70	1.00
5.45	1.15 ± 0.10	0.92 ± 0.04	802.6 ± 107.1	11.95	1.00
6.00	1.05 ± 0.09	0.91 ± 0.04	861.9 ± 112.9	12.84	0.97
6.49	1.09 ± 0.10	0.93 ± 0.04	851.4 ± 118.4	12.69	1.00
7.00	1.27 ± 0.14	0.92 ± 0.05	721.3 ± 107.8	10.75	1.00
7.50	1.64 ± 0.19	0.87 ± 0.06	528.7 ± 97.8	7.88	0.97
7.63	2.21 ± 0.11	0.88 ± 0.03	398.2 ± 32.6	5.93	1.04
8.50	3.37 ± 0.21	0.83 ± 0.04	247.2 ± 26.4	3.68	1.08
8.93	4.10 ± 0.25	0.63 ± 0.03	153.7 ± 16.2	2.29	0.87

[a] For experimental details, see Mole and Horton (1973a); enzyme concentration, 0.20–0.40 μM.

analyzed in terms of a dominant tryptophyl residue which is susceptible to oxidation by *N*-bromosuccinimide (Steiner, 1971).

Unfortunately, interpretation of the role of tryptophyl residues in the catalytic activity of papain has been hampered by the possibility of accompanying modifications of other residues, including Cys-25, and by conflicting conclusions regarding the nature of the environment of the tryptophans involved. Accordingly, the development of 2-chloromethyl-4-nitrophenyl (*N*-carbobenzoxy)glycinate (Z-Gly-ONB-Cl)[1] as an active-site-directed, tryptophan-selective reagent for papain (Mole and Horton, 1973b) has provided a means for investigating the kinetic consequences of modifying a tryptophan vicinal to the enzyme's sulfhydryl group.

Experimental Procedure

Materials. 2-Hydroxy-5-nitrobenzylated papain was prepared as described in the preceding paper (Mole and Horton, 1973b). BzArgOEt (lot 81C-0060) and ethylene diamine tetraacetate were purchased from Sigma Chemical Co. BzArgNan·HCl (lot 72046), mp 223°, was obtained from the Protein Research Foundation, Peptide Institute, Osaka, Japan. β-Mercaptoethanol was purchased from Matheson Coleman and Bell.

Methods. The kinetics of papain-catalyzed hydrolysis of BzArgNan were measured by continuously monitoring the release of *p*-nitroaniline at 410 nm as previously described (Mole and Horton, 1973a). Hydrolysis of BzArgOEt was measured spectrophotometrically at 285 nm (Whitaker and Bender, 1965). Catalytic coefficients, k_{cat}, and apparent Michaelis constants, K_m(app), were evaluated by computer fitting to the hyperbolic Michaelis–Menten equation according to an adaptation of the program of Cleland (1967). The rate constants governing enzyme acylation and deacylation, k_2 and k_3, respectively, and the ionization constants for two functional groups in papain's catalysis, K_1 and K_2, were evaluated by the procedure previously described (Mole and Horton, 1973a).

Results

The kinetic constants characterizing the HNB-papain-catalyzed hydrolysis of benzoyl-L-arginine ethyl ester and benzoyl-L-arginine *p*-nitroanilide at pH 6.5 were evaluated and compared with those of the native papain-catalyzed reactions. Results are given in Table I. Hydroxynitrobenzylation of the enzyme through its reaction with Z-Gly-ONB-Cl led to a 24% increase in the bimolecular rate constant, k_{cat}/K_m(app), of BzArgOEt hydrolysis and a 240% increase in that of the corresponding *p*-nitroanilide. These increases in enzymatic activity resulted in part from decreases in the K_m(app) for each substrate; however, hydroxynitrobenzylation led to a reduction in k_{cat} for the ester by 28% and an increase in k_{cat} for the anilide of 27%.

Inasmuch as most active-site modifications result in losses in enzymatic activities, we undertook a detailed steady-state kinetic analysis of HNB-papain's enhanced catalytic activity toward BzArgNan. The resulting kinetic parameters, k_{cat}, K_m(app), and k_{cat}/K_m(app), are recorded as a function of pH in Table II, and plotted in Figure 1 together with the corresponding values for native papain. Whereas a plot of k_{cat}/K_m(app) as a function of pH for native papain produced a bell-shaped curve whose ascending and descending limbs were governed by pK_a values of 4.3 and 8.2, respectively (Mole and

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[1] Abbreviations used are: HNB, 2-hydroxy-5-nitrobenzyl; Z-Gly-ONB-Cl, 2-chloromethyl-4-nitrophenyl (*N*-carbobenzoxy)glycinate; BzArgOEt, *N*-benzoyl-L-arginine ethyl ester; BzArgNan, *N*-benzoyl-L-arginine *p*-nitroanilide.

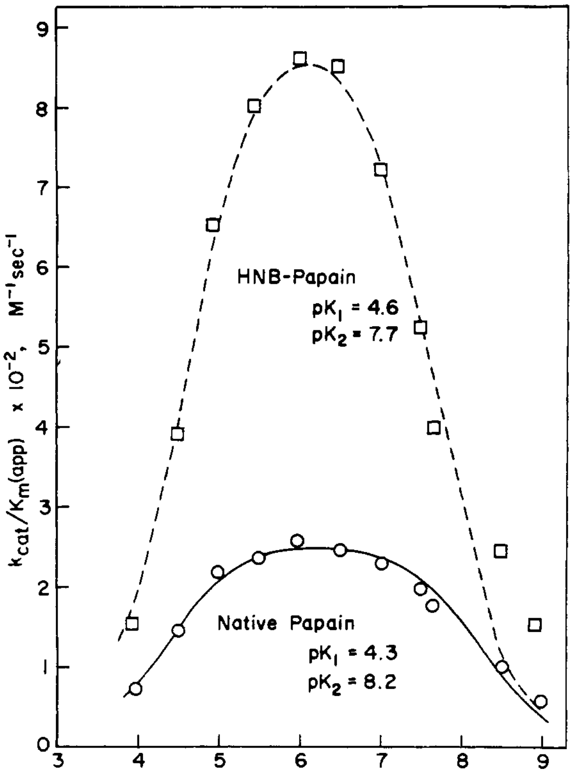

FIGURE 1: Comparison of k_{cat}/K_m (app) *vs.* pH profiles of papain and HNB-papain for BzArgNan. The lines represent the computer-generated curves which provide the best fit of the data to the ionizations of two functional groups on each enzyme (see Mole and Horton, 1973a).

Horton, 1973a), a similar plot of the data for HNB-papain revealed $pK_1 = 4.6 \pm 0.1$ and $pK_2 = 7.7 \pm 0.2$.[2]

Plots of $1/K_m$(app) *vs.* k_{cat}/K_m(app) (Figure 2) and k_{cat} *vs.* K_m(app) (Figure 3) provided K_s values of 14.77 and 14.90 mM, respectively, and k_3(lim) values of 1.0 sec^{-1} for the HNB-papain-catalyzed hydrolysis of BzArgNan. Individual values for the rate constants governing acylation, k_2, and deacylation, k_3, are recorded as a function of pH in Table II, and plotted in Figure 4.

The resulting k_2(lim) for HNB-papain is 13.2 sec^{-1}, as compared to 1.85 sec^{-1} for native papain.

Discussion

Hydroxynitrobenzylation of papain through its reaction with Z-Gly-ONB-Cl results in enhanced catalytic activity. Toward the ester substrate, BzArgOEt, the catalytic efficiency of HNB-papain, k_{cat}/K_m(app), is 24% higher than that of native papain; this is accounted for by a lowering of the K_m(app) from 16.9 to 9.8 mM, accompanied by a 28% decrease in k_{cat}. Toward BzArgNan, however, HNB-papain shows a 240% increase in catalytic activity over native enzyme (Figure 1), which is a result of both a decrease in K_m(app), from 2.95 to 1.09 mM, and a 27% increase in k_{cat}.

A detailed examination was made of the steady-state kinetics of HNB-papain's action on BzArgNan, according to

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{P_1}{\underset{+}{ES'}} \xrightarrow[H_2O]{k_3} \underset{P_2}{\underset{+}{E}} \qquad (1)$$

where P_1 and P_2 are *p*-nitroaniline and *N*-benzoyl-L-arginine, respectively, formed by hydrolysis of the substrate, S; ES is the Michaelis complex; and ES′ is the acylated enzyme intermediate.

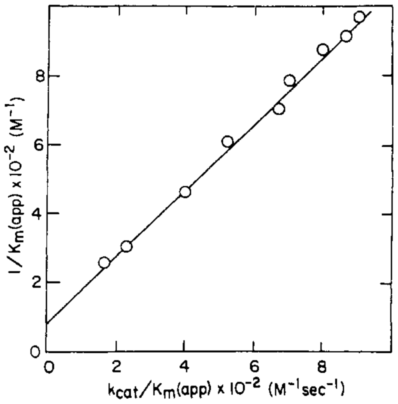

FIGURE 2: The variation of $1/K_m$(app) with k_{cat}/K_m(app), plotted for pH values above 4.94. The line represents the computer fit of the data to the relationship, $1/K_m(\text{app}) = 1/K_s + (k_{cat}/K_m(\text{app}))/k_3$.

Kinetic relationships for this mechanism are

$$K_m(\text{app}) = k_3(k_{-1} + k_2)/(k_2 + k_3)k_1 \approx [k_3/(k_2 + k_3)]K_s \qquad (2)$$

and

$$k_{cat} = k_2k_3/(k_2 + k_3) \qquad (3)$$

Such kinetic evaluation revealed that hydroxynitrobenzylation of the enzyme had resulted in: a twofold increase in the apparent value of K_s, from 7.9 to 14.9 mM; a sevenfold increase in the rate constant governing enzyme "acylation," k_2; and slight changes in the ionizations of two functional groups involved in the "acylation" step, from pK_a values of 4.3 and 8.2 to 4.6 and 7.7; but no significant change in "deacylation" rate, k_3 nor in the pK_a of the functional group involved in deacylation, which remained 3.9. One effect of the hydroxynitrobenzyl residue in the HNB-papain-catalyzed hydrolysis of BzArgNan may thus be to facilitate the rate of enzyme acylation by increasing k_2 [and thereby the apparent value of K_s which is actually $(k_{-1} + k_2)/k_1$].

It is apparent that the simple acylation–deacylation mechanism of eq 1 does not adequately depict the action of papain on the *p*-nitroanilide of *N*-benzoyl-L-arginine (Mole

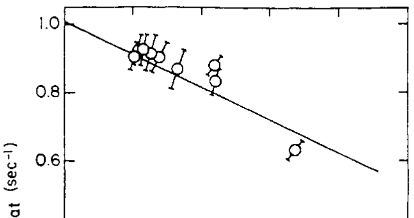

FIGURE 3: The variation of k_{cat} with K_m(app) for pH values above 4.94. The line represents the computer fit of these data to the equation, $k_{cat} = k_3 - [k_3K_m(\text{app})/K_s]$.

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[2] The computer-evaluated ionization constants and standard errors were: $K_1 = 25.0 \pm 7.1 \times 10^{-6}$; $K_2 = 21.4 \pm 9.4 \times 10^{-9}$.

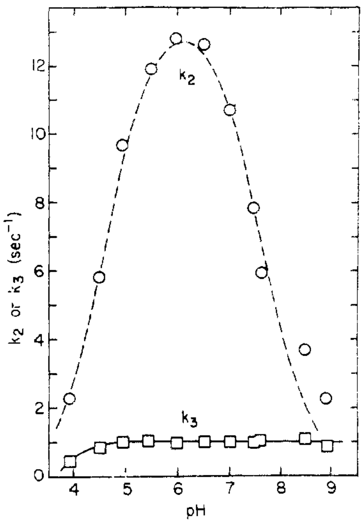

FIGURE 4: Variation of acylation and deacylation rate constants of HNB-papain for BzArgNan with pH. The lines represent the computer-generated curves which fit the k_2 data to the ionizations of two functional groups, and the k_3 data to the ionization of a single group (see Mole and Horton, 1973a).

and Horton, 1973a). Brocklehurst *et al.* have demonstrated that the kinetic consequences of nonproductive binding of such acylamino acid substrates would be to provide a low estimate for the rate constant governing acylation (k_2) by the Whitaker and Bender (1965) analysis used in the present study; however, this procedure would provide the correct value for k_3(lim) under such circumstances (Brocklehurst *et al.*, 1968). Thus, the finding that k_3(lim)(anilide) is not equal to or greater than k_{cat}(ester) (Table I) reflects the earlier observation that the k_3(lim) of papain for BzArgOEt is 30 sec^{-1} whereas the k_3(lim) for BzArgNan is only 1 sec^{-1} (Mole and Horton, 1973a). Thus, to account for the apparent k_3(lim) of 1 sec^{-1} assuming an actual deacylation rate constant of 30 sec^{-1} were to exist for the anilide as well as the ester, "wrong-way" binding of the arginyl substrate would have to occur in a special manner which would permit a very slow deacylation of wrong-way acylated papain. Alternatively, the simple three-step mechanism of eq 1, which has been shown to apply to the papain-catalyzed hydrolysis of a variety of ester substrates, may not accurately reflect the mechanism of anilide hydrolysis by either the native or the hydroxynitrobenzylated enzyme. It is nonetheless apparent that it is the "k_2" portion of k_{cat} which is augmented by hydroxynitrobenzylation of papain, and not the "k_3"-controlled deacylation step(s).

The effect of hydroxynitrobenzylation on k_2 contrasts with the rate enhancement observed when acridine dyes such as proflavine are added to papain. Hall *et al.* (1972) observed that proflavine interacted with papain to lower its K_m(app) for BzArgOEt and for *N*-benzoylglycine ethyl ester, without significantly affecting k_{cat} for either substrate; and concluded that the effect was likely a manifestation of an increase in enzyme–substrate association (k_1) or a decrease in dissociation of the ES complex (k_{-1}). Essentially the opposite effects had been reported by Hollaway (1968) for the proflavine-induced increase in the catalytic activity of the sulfhydryl enzyme, ficin, toward BzArgOEt. In that case, an increase in k_{cat} was observed without significant alterations in K_m(app), and the results were interpreted as an indication that acylation of the enzyme was facilitated by dye binding. Lack of enhancement of the rate of ficin-catalyzed hydrolysis of *p*-nitrophenylhippurate by proflavine was cited as evidence in support of the dye's effect on acylation, k_2, since deacylation is known to be the rate-limiting step in hydrolysis of that substrate.

Another instance of enhanced catalytic activity has been reported by Kirschenbaum (1971). Treatment of inactive papain with a 6:1 ratio of *N*-bromosuccinimide led to a preparation which, when activated by mercaptan, showed increased activity toward *N*-benzoyl-DL-arginine *p*-nitroanilide. The causes of the enhanced activity were not elucidated. The possibility that *N*-bromosuccinimide treatment had converted some of the "irreversibly" inactivated papain in the crude preparation to a mercaptan-activatable species was recognized (Kirschenbaum, 1971), although the possible loss of inhibition of the modified enzyme by the D isomer (Mole and Horton, 1973a) was not discounted. However, in light of the present findings with *fully* active papain, in which hydroxynitrobenzylation of a tryptophyl residue facilitates enzyme acylation by the L isomer of BzArgNan through a sevenfold increase in k_2, it seems likely that oxidation of a tryptophyl residue by *N*-bromosuccinimide treatment may have similarly enhanced the acylation rate in Kirschenbaum's study.

Final evaluation of the electronic environment of the hydroxynitrobenzyl group and the mechanism by which it facilitates enzyme acylation in the catalytic action of HNB-papain must await elucidation of its position in the primary sequence of the enzyme. Nevertheless, it is of interest to summarize the conclusions which may be drawn from the spectral and kinetic characteristics of HNB-papain. The spectral sensitivity of the HNB reporter group to pH (Mole and Horton, 1973b) suggested that its phenolic hydroxyl group is neither hydrogen bonded to another residue in the protein nor buried in a hydrophobic environment, but protrudes into the aqueous solvent. However, the "nitro side" of the reporter group may be at least partially buried in the enzyme's structure, since a degree of dissymmetry in the group's electronic environment is manifested by the extrinsic Cotton effects observed in the visible spectrum. Barel and Glazer (1969) have observed that neither enzyme activation nor active site alkylation affected the ultraviolet circular dichroism of native papain, nor the solvent perturbation of its difference spectra or fluorescence spectra. In contrast, absorption spectra and circular dichroism of the hydroxynitrobenzyl reporter group in HNB-papain are sensitive to enzyme activation, alkylation of the active site, and interaction with reversible inhibitors (Mole and Horton, 1973b). When the procedure by which the HNB group was incorporated into papain, through the enzyme's reaction with active-site-directed Z-Gly-ONB-Cl, and the kinetic enhancement of the modified enzyme's activity toward BzArgNan are taken into account, it seems highly probable that the HNB group resides in close proximity to the active site. Once the position of the HNB group in the primary structure of papain becomes established, it will be interesting to compare its orientation with that established for the appropriate tryptophyl residue in the crystalline state (Drenth *et al.*, 1968, 1970, 1971) and with the environment inferred from photooxidation of papain in solution (Jori and Galiazzo, 1971).

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Behavior of Chloramphenicol-Induced Phenylalanine Transfer Ribonucleic Acid during Recovery from Chloramphenicol Treatment in *Escherichia coli*†

Michael B. Mann‡ and P. C. Huang*

ABSTRACT: Additional chromatographic forms of phenylalanine tRNA appear in *Escherichia coli* incubated with chloramphenicol. The kinetics of change in the level of these forms is examined during the period of recovery from this treatment. The results are consistent with a model relating all of the forms by a simple conversion pathway.

It is known that *Escherichia coli* treated with high levels of chloramphenicol (50–200 μg) continue to synthesize tRNA, although cell growth and protein synthesis are inhibited (Kurland and Maaløe, 1962; Ezekiel and Valulis, 1965; Waters, 1969). It has been further shown that most of the chloramphenicol-induced tRNA of the leucine and phenylalanine acceptor type differs chromatographically from the respective normal form(s) found in exponentially growing cells (Waters, 1969).

It was pointed out by Waters (1969) that the chloramphenicol-induced tRNA forms could conceivably represent intermediates in the maturation process of tRNA biosynthesis. This possibility is supported here by a kinetic analysis of the change occurring in the levels of each of the chromatographic forms of phenylalanyl-tRNA during the period of recovery from chloramphenicol treatment. The results are consistent with a model relating all of the forms by a simple conversion pathway.

Materials and Methods

Escherichia coli THU (Stern *et al.*, 1964), requiring thymidine, histidine, and uracil, was used throughout this study. Minimal salts medium modified from Kurland and Maaløe (1962) contained, per liter: tris(hydroxymethyl)aminomethane base (Tris), 12 g; potassium chloride, 2 g; ammonium chloride, 2 g; magnesium chloride hexahydrate, 0.5 g; sodium sulfate, 20 mg; and sufficient concentrated HCl to bring the pH to 7.2. To this was added 0.02% vitamin-free casein hydrolysate (casamino acids) (General Biochemicals Corp.); L-histidine, 20 μg/ml; thymidine, 10 μg/ml; and uracil, 20 μg/ml. The carbon source was 0.5% glycerol. Inorganic orthophosphate in the form of disodium hydrogen phosphate dihydrate was 1 mM.

Reagents used in this study were obtained from the following sources: deoxyribonuclease I (DNase) electrophoretically purified (lot No. X-1862) from Schwarz/Mann; chloramphenicol (lot 91C-0680) from Sigma Chemical Co.; Plaskon CTFE, 2300 powder (lot No. 24537F) from Allied Chemical Corp.; Adogen 464, [C_8–C_{10}] trialkylmethylammonium chloride (lot 117-M644-640) from Ashland Chemical Co.; Whatman DE-52 grade of diethylaminoethylcellulose (control No. 2452/69) from W. & R. Balston, Ltd.; glass beads, 200-μ diameter, from 3M Corp.; Triton X-100 from Rohm & Haas; Millipore filters, HA 0.45 μ, 47 mm, from Millipore Filter Corp.; and 2,5-diphenyloxazole and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene from Research Products International Corp. All other chemicals were reagent grade.

Radioactive compounds were obtained from the following sources: L-[^{14}C]phenylalanine (U) 255 Ci/mol from Schwarz/Mann; L-[^{3}H]phenylalanine (N) 12.8 Ci/mmol from New England Nuclear.

Frozen pellets of *E. coli* B cells, harvested in early log phase (lot No. 55462), were from General Biochemicals Corp.

Growth of Cells. Cell growth was followed turbidimetrically in side-arm tubes and flasks with a Coleman Junior spectrophotometer at 650 nm. Reported optical density (OD_{650}) measurements refer to readings made in 13 mm × 100 mm test tubes. An OD_{650} of 0.1 corresponded to 4×10^8 cells/ml.

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