Time-Dependent Inhibition of *Bacillus stearothermophilus* Alanine Racemase by (1-Aminoethyl)phosphonate Isomers by Isomerization to Noncovalent Slowly Dissociating Enzyme–(1-Aminoethyl)phosphonate Complexes†

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ABSTRACT: An alanine racemase encoded by a gene from the thermophilic Gram-positive bacterium *Bacillus stearothermophilus* is overproduced to 0.3% of the soluble protein when carried on plasmid pICR4 in *Escherichia coli* [Inagaki, K., Tanizawa, K., Badet, B., Walsh, C. T., Tanaka, H., & Soda, K. (1986) *Biochemistry* (third paper of four in this issue)]. Purification of large quantities (50 mg) of racemase permits study of time-dependent inactivation by D and L isomers of the antibacterial (1-aminoethyl)phosphonate (Ala-P), the phosphonate analogue of alanine. The time-dependent activity loss by this compound now appears general to Gram-positive but not to Gram-negative racemases [Badet, B., & Walsh, C. (1985) *Biochemistry 24*, 1333] and is shown to occur by extremely slow dissociation of a noncovalent E·Ala-P complex. Ala-P binds initially in a weak, reversible (K_I = 1 mM) competitive manner but is slowly isomerized (k_{inact} = 6–9 min^{-1}) to a stoichiometric enzyme complex, which in turn dissociates extremely slowly, with a half-time about 25 days. Thus, Ala-P is a slow but not a tight-binding inhibitor. The E·Ala-P complex is not reducible by borohydride but does perturb the fluorescence of bound pyridoxal 5′-phosphate coenzyme. Determination of the sequence of an active site octapeptide of the *B. stearothermophilus* alanine racemase shows homology with the sequence of a Gram-negative *Salmonella typhimurium* alanine racemase that is not susceptible to time-dependent inhibition by Ala-P. Studies with Ala-P analogues suggest the phosphonate dianion is crucial for stable formation of an isomerized long-lived E·Ala-P-inhibited complex.

The peptidoglycan layer of the bacterial cell wall is one of the major rigid structures protecting bacteria against osmotic shock and cell lysis. The alanine branch of peptidoglycan biosynthesis comprises three enzymatic steps. Alanine racemase converts L-Ala to D-Ala. D-Ala-D-Ala ligase generates the D-Ala-D-Ala dipeptide, which is then attached to the growing UDP–muramyl tripeptide intermediate by D-Ala-D-Ala adding enzyme. Alanine racemase has thus been identified as the first enzyme committed to cell wall biosynthesis. As such, and because it is restricted to procaryotes, the racemase has been a target for antibacterial drugs. In fact there are at least two genes encoding for alanine racemases in *Salmonella* typhimurium (Wasserman et al., 1983). This may reflect the fact that alanine racemization is actually a branch point in alanine metabolism, providing D-alanine not only for cell wall constitution but also as an energy source for growth. The *Salmonella* genes map at distinct genetic loci, and their specific regulation and physiological roles are under study.

Gram-negative and Gram-positive bacteria are often differentially susceptible to antibiotics. We have recently purified alanine racemases from both bacterial types for comparison of molecular susceptibility to the alanine analogue (1-aminoethyl)phosphonic acid (Ala-P),[1] the phosphonate analogue of alanine. Our recent purification and characterization of an alanine racemase from *Streptococcus faecalis* (in Badet & Walsh, 1984) was motivated, in part, by a report from the Roche–Welwyn group (Atherton et al., 1979; Allen et al., 1979) that in crude extracts this Gram-positive racemase showed time-dependent inhibition by Ala-P, the active component derived by intracellular peptidase cleavage of the antibacterial alaphosphin (L-Ala-L-Ala-P). We confirmed this effect with pure racemase from that source and showed that inhibition derived from slow dissociation of Ala-P tightly, but noncovalently, bound to enzyme. Because we had to purify the *Sr. faecalis* several thousandfold to homogeneity, little protein was available for detailed mechanistic work. Gram-negative alanine racemases are not susceptible to time-dependent inhibition by Ala-P.

In this paper we deal with the alanine racemase purified from the thermophilic Gram-positive *Bacillus stearothermophilus*. This racemase gene has been cloned into *Escherichia coli* on a plasmid and its overproduction and purification are described in the preceding paper (Inagaki et al., 1985). With this pure, Gram-positive-derived alanine racemase available in quantity, we report here its susceptibility to Ala-P inactivation and describe more complete characterization of its dramatic slow dissociation behavior.

Experimental Procedures

Materials

L- and D-alanine, *B. subtilis* L-alanine dehydrogenase (30 units/mg in 50% glycerol), CHES, HEPES, PLP were purchased from Sigma. Hog muscle lactate dehydrogenase (550 units/mg, glycerol solution) was from Boehringer. D- and L-Ala-P were purchased from Fluka. [U-^{14}C]-L-Ala-P was

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[1] Abbreviations: Ala-P, (1-aminoethyl)phosphonic acid; PNP, pyridoxyl; PLP, pyridoxal 5′-phosphate; L-ADH, L-alanine dehydrogenase; DAAO, D-amino acid oxidase; LDH, L-lactate dehydrogenase; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; d, doublet; t, triplet; m, multiplet; ppm, part per million.

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kindly provided by Dr. C. Hassal (Roche Products Ltd., U.K.). β-Fluoro-Ala-P was kindly provided by Merck Sharp & Dohme. We are indebted to Pr. F. Le Goffic for providing us with the β-chloro-substituted Ala-P.

Methods

N-Phosphopyridoxyl-L-Ala and N-phosphopyridoxy-L-Ala-P were synthesized as described (Badet & Walsh, 1984).

Dimethyl N-Carbobenzyloxy(1-aminoethyl)phosphonate. Diphenyl *N*-carbobenzyloxy(1-aminoethyl)phosphonate (Oleksyszyn et al., 1979) (2.05 g, 5 mmol) in methanol (40 mL) was treated with sodium methoxide (60 mg, 11 mmol) at room temperature; TLC (silica, $CHCl_3$) showed disappearance of the starting material after 10 h. The solvent was removed, the oil was dissolved in CH_2Cl_2, and the solution was washed to neutrality and dried. The compound was purified from contaminating phenol by flash chromatography (silica; $CHCl_3$/AcOEt, 1:1), giving an oil (1.37 g, 95%) pure by TLC: ^1H NMR ($CDCl_3$) δ 7.32 (5 H, s), 5.83 (NH, d, 10 Hz), 5.1 (2 H, s), 4.28 (1 H, m), 3.72 and 3.65 (6 H, d, J_{HP} = 10.5 Hz), 1.37 (3 H, dd, J_{HH} = 7 Hz, J_{HP} = 17 Hz).

Dimethyl (1-aminoethyl)phosphonate was obtained in quasi-quantitative yield by transhydrogenation with cyclohexene in methanol (Anantharamaiah & Sivanandaiah, 1977): ^1H NMR (D_2O) δ 3.71 (6 H, d, J_{HP} = 10.3 Hz), 3.16 (1 H, m), 1.2 (3 H, dd, J_{HH} = 7 Hz, J_{HP} = 18 Hz).

Monomethyl (1-Aminoethyl)phosphonate. The oil (0.61 g, 4 mmol) was treated with 4 mL of 1 M NaOH at room temperature for 2 h; TLC (*n*-BuOH/AcOH/H_2O, 6:2:2; ninhydrin visualization) showed complete conversion of the starting material: ^1H NMR (D_2O) δ 3.45 (3 H, d, J_{HP} = 10.3 Hz), 2.76 (1 H, m), 1.08 (3 H, dd, J_{HH} = 7 Hz, J_{HP} = 16.5 Hz). The monoester of Ala-P migrated on TLC between free Ala-P and the dimethyl ester. The monosodium salt was stored as a desiccated hygroscopic powder.

Assays. The racemase spectrophotometric assay using either DAAO coupled to LDH or L-ADH has been already described (Badet & Walsh, 1984). A unit of activity is defined as 1 μmol of substrate epimerized per minute. In the L to D direction, assays could be performed at pH 7.4 or 9; in the D to L direction, however, assays could not be performed at pH 7.4 because of the low efficiency of the L-ADH coupling enzyme at this pH.

Kinetic Analysis. All inactivation experiments were conducted at pH 7.4 in 100 mM HEPES. Methods for analysis of the slow binding of Ala-P were adapted from Cha (1975, 1976), Shapiro and Riordan (1984), and Morrison and Stone (1984). A 1-mL cuvette containing a mixture of inhibitor, substrate, DAAO (16 units), LDH (0.2 mg), and NADH was equilibrated at 37 °C for at least 15 min inside the spectrophotometer. The assay was then begun by addition of enzyme; with these amounts of coupling enzymes, there was no lag in the reaction. A pseudo-first-order constant (k_{obsd}) for the decrease in reaction velocity was calculated by using the equation $v = v_s + (v_0 - v_s) \exp(-k_{obsd}t)$, where v_0, v, and v_s are velocities measured at time zero, t, and steady state, respectively. The decrease in absorbance at 340 nm was continuously monitored; changes in absorbance during successive equal time intervals were measured as approximations of the reaction velocity (arbitrary units). k_{obsd} was determined from the slope of a plot of ln $(v - v_0)$ vs. time. Individual rate and inhibition constants were calculated from k_{obsd} values as described below.

Time-dependent inhibition and steady-state analyses were performed after dilution in 100 mM HEPES, pH 7.4, as described under Results. Constants resulting from double-reciprocal plots were calculated by the v^4 weighting method of Wilkinson (1961); other plots were fitted to a regression analysis program.

Stoichiometry of Labeling. Inactivation with radiolabeled L-Ala-P (6.25 Ci/mol) was performed in the same way as previously described for the *Sr. faecalis* enzyme (Badet & Walsh, 1984). This solution was used to follow the regain of activity (see below) and to analyze the product released by heat treatment or borohydride reduction.

Fluorescence Studies. PLP concentration was determined by fluorescence as described by Adams (1979). Modifications in the enzyme fluorescence emission spectrum during inactivation with Ala-P are described under Results.

Protein Determination. Protein concentrations were estimated from absorbance at 280 nm or by the method of Lowry (1951) or Bradford (1976) and corrected from the results of amino acid analysis by the following (×): 0.854, 0.996, and 0.765, respectively.

HPLC. HPLC separations of PNP–amino acid adducts were performed isocratically on a strong anion exchange column (Partisil SAX, Whatman) and either with 250 mM ammonium formate, pH 3.5 (PNP–Ala-P), or 50 mM KPO_4, pH 3.5 (PNP–Ala), as solvent; detection was at 313 nm, and the flow rate was 1 mL/min.

Active Site Sequence. NaB^3H_4 reduction of pure enzyme (5 mg), reductive alkylation, trypsin digestion, and HPLC peptide purification were accomplished as described for the *Sl. typhimurium dadB* alanine racemase (Badet et al., 1984).

Results

Purification and Characterization of the B. stearothermophilus Alanine Racemase. With the gene for *B. stearothermophilus* cloned on plasmid pICR 4 and expressed in *E. coli* C600, 0.3% of the soluble protein is the thermophilic Gram-positive racemase. This has facilitated its purification to homogeneity as described in the preceding paper (Inagaki et al., 1986) and readily permits accrual of 50–100-mg quantities of pure enzyme. However, we have seen some irreproducibility in PLP coenzyme content. Some preparations contain two PLP/dimer while other preparations of pure enzyme, such as that used below in the [^{14}C]Ala-P inactivation study, contain only one PLP/two subunits. The reason for variable loading on overproduction is not yet clear, but PLP content was carefully determined for each preparation.

Inactivation Studies with Ala-P. Initial velocities studies were initiated by addition of enzyme (92 pM) to solutions containing varying concentrations of L-alanine and fixed concentrations of L-Ala-P. Competitive inhibition ensued with a K_i = 0.8 mM (data not shown). If, on the other hand, the racemase (9.4 nM) was preincubated with varying concentrations of L-Ala-P for 24 or 75 h and then assayed after 100-fold dilution in substrate, the apparent inhibition constant dropped 3 orders of magnitude (K_i = 1.2 μM), and the noncompetitive pattern observed by Lineweaver–Burk analysis (data not shown) is a result of only free enzyme reacting with substrate. Increasing amounts of Ala-P in the preincubation result in decreasing amounts of free enzyme with concomitant decrease in apparent V_{max}. Further, the K_m of the enzyme after preincubation is identical with that of native enzyme, suggesting only unmodified enzyme is catalytically competent. There is no dissociation of E·Ala-P in the time course of the catalytic assay (ca. 10–15 min). Therefore, an extremely long-lived enzyme–Ala-P complex would account for this kinetic behavior (Segel, 1975).

Such a pattern was recently described qualitatively with the purified alanine racemase from another Gram-positive bacterium *Sr. faecalis* (Badet & Walsh, 1984). In confirmation,

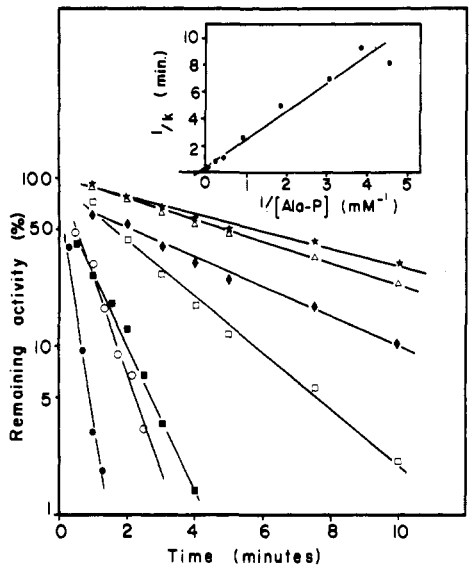


FIGURE 1: Time-dependent inhibition of *B. stearothermophilus* alanine racemase by L-Ala-P. The racemase (375 nM) was incubated in 100 mM HEPES, pH 7.4, with 0.26 (*), 0.33 (△), 0.55 (♦), 1.11 (□), 2.17 (■), 4.2 (○), or 10 mM (●) concentration of L-Ala-P. At different time points, aliquots were diluted 100-fold at 4 °C in the same buffer, and the remaining activity was checked in D to L direction at 100 mM D-alanine in 100 mM CHES, pH 9, with 20 μL of the diluted solution as described under Assays. k is obtained from the slope of the lines. (Inset) Double-reciprocal plot $1/k$ vs. $1/$[L-Ala-P].

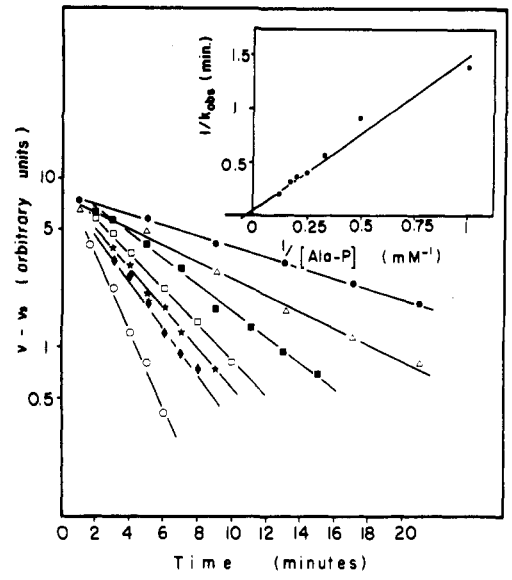


FIGURE 2: Determination of k_{obsd} for the slow onset of inhibition of *B. stearothermophilus* alanine racemase by L-Ala-P. Assays were initiated by addition of the enzyme (92 pM final) to preincubated mixtures of 20 mM L-alanine, NADH, and coupling enzymes in 100 mM HEPES, pH 7.4, containing 1 (●), 2 (△), 3 (■), 4 (□), 5 (*), 6 (♦), and 10 mM (○) L-Ala-P. k_{obsd} is obtained from the slope of the lines. (Inset) Dependence of $1/k_{obsd}$ on $1/$[Ala-P].

incubation of *B. stearothermophilus* alanine racemase with [U-^{14}C]-L-Ala-P, followed by gel filtration chromatography, produced an inactive protein peak containing ^{14}C stoichiometric with the PLP cofactor (in this particular preparation, 0.5 mol/mol of subunit, vide supra). That this enzyme-associated radioactivity was intact, noncovalently attached [U-^{14}C]-Ala-P was demonstrated by 3-min heating, precipitation of unlabeled protein, and cochromatography of the radioactive material in the supernatant with authentic Ala-P by HPLC. As in the *Sr. faecalis* case, cocrystallization to constant specific radioactivity also occurred. Finally, a parallel behavior of initial competitive inhibition but noncompetitive inhibition at steady state has been detected with deoxycoformycin interaction with adenosine deaminase in what has become a classic example of slow, tight-binding noncovalent enzyme inhibition (Cha et al., 1975).

Analysis of Time-Dependent Inhibition. For slow binding inhibitors, Cha (1975, 1976) and Morrison (1984) among others have distinguished between an initial slow binding process (mechanism A) and a process with typical rapid

mechanism A

$$E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI$$

mechanism B

$$E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^*$$

mechanism C

$$E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E^* \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E^*I$$

formation of an EI complex followed by a slow isomerization process to a slowly dissociating EI* complex. A third variant, mechanism C, has slow isomerization of E to E* prior to binding of inhibitors in an E*I complex, which is slow to dissociate to E + I. The most likely general case is mechanism B and that appears to apply for Ala-P and the *B. stearothermophilus* alanine racemase as documented below.

Figure 1 shows the time-dependent pseudo-first-order loss of activity on incubation of 375 nM enzyme with the indicated concentrations of L-Ala-P followed by dilution after varying periods of time into 100 mM D-alanine containing buffer to assay residual activity. The inset shows a double-reciprocal plot yielding a limiting k_{inact} of 9.6 ± 1.7 min^{-1} and an apparent $K_i' = 22.8 \pm 4.7$ mM. Since this $K_i' = K_i(1 + [S]/K_m)$, we could obtain the initial $K_i = 0.93$ mM in good agreement with the value above. From the values of k_{obsd} for inactivation at any concentration of L-Ala-P in Figure 1 and similar experiments (data not shown), we can calculate the net second-order constant k_1 (in mechansim A, B, or C) and find a value $k_1 = 6$–8 M^{-1} s^{-1}. By contrast, Cha et al. (1975) found a value of 2×10^6 M^{-1} s^{-1} for coformycin and adenosine deaminase. While 10^6 M^{-1} s^{-1} is a credible value for an association rate constant of enzyme and ligand, 10 M^{-1} s^{-1} is not and suggests mechanism B is in fact insufficient. Perhaps, two isomerization complexes (mechanism D) are required, and the net k_1 mea-

mechanism D

$$E + I \rightleftarrows EI \rightleftarrows EI' \overset{\text{slow}}{\rightleftharpoons} EI''$$

sured above is net conversion of E + I to EI'. In any case, we feel this exceedingly low value for k_1 is diagnostic of an additional step. Cleland and co-workers (Schloss & Cleland, 1982) found a value of 1050 M^{-1} s^{-1} for a net k_1 in inhibition of isocitrate lyase by 3-nitropropionate nitronate anion, and that value is also anomalously low.

Next, the time course of inactivation by L-Ala-P (1–10 mM) in the presence of the substrate L-alanine (20 mM) was analyzed by initiation with racemase (92 pM) and monitoring of the NADH decrease in the DAAO, NADH, LDH-coupled assay. Figure 2 shows plots of log $(v - v_s)$ vs. time. We could not readily obtain limiting v_s at low Ala-P concentration and assumed this velocity was zero, on the basis of all other evidence above and below on complete inactivation and extremely slow regain of activity on slow dissociation. The K_{obsd} values of Figure 2 have the form indicated in Table I if mechanism A, B, or C obtains (for mechanism D, one can lump prior steps into EI formation and k_1 and k_2 become composite constants

Table I: Relationship for Apparent First-Order Constant Associated with Different Mechanisms

mechanism	k_{obsd}
A	$k_1[I]/(1 + [S]/K_m) + k_2$
B	$k_3[I]/(K_i' + [I]) + k_4$[a]
C	$k_1/(1 + [S]/K_m) + k_2k_i'/([I] + K_i')$[b]

[a] $K_i' = k_2/k_1$. [b] $K_i' = K_i(1 + [S]/K_m)$. $K_i = k_4/k_3$.

in this variant of mechanism B).

For mechanism A, the initial velocity would be independent of inhibitor concentration, which is not the case. For mechanism C, k_{obsd} decreases as [I] increases while we see k_{obsd} increases with increasing [I] as projected by mechanism B. A further distinction of mechanism A vs. B is to plot $1/(k_{obsd} - k_2)$ or $1/(k_{obsd} - k_4)$ vs. $1/[I]$. While linear in both cases, in mechanism A the line passes through the origin while with B it has a vertical intercept at $1/k_3$ and a horizontal intercept at $-1/K_i'$. Since k_4 or k_2 (in mechanism A) is negligible, as shown in Figure 2, a plot of $1/k_{obsd}$ vs $1/[I]$ gives $k_3 = 2.5$ min^{-1} and $K_i = 6.9$ mM, in comparison with 10 min^{-1} from figure 1 and 1 mM for K_i as noted earlier. The key fact is that again mechanism A is ruled out. One can also plot $1/(k_{obsd} - k_4)$ vs. [S] at constant [I]. This experiment conducted at 5 mM L-Ala-P with variation of [S] from 10 to 75 mM gave a horizontal intercept of 16 mM (data not shown); this value is too high to be K_m, which should be obtained if mechanism A were followed. For mechanism B, this intercept corresponds to $K_m([I] + K_i)/K_i$ and yields $K_i = 1.8$ mM, again assuming $k_4 = 0$ for these plots. This 1.8 mM value is in good agreement with that obtained from Lineweaver–Burk analysis described above.

Given the formal mechanism B, v_0 is given by

$$v_0 = \frac{V_m[S]}{K_m(1 + [I]/K_i) + [S]}$$

which predicts a linear relationship between $1/v_0$ and [I] with slope $K_m/([S]V_mK_i)$. The experiment conducted at [S] = 20 mM gave a linear plot (data not shown) for which a $K_i = 1.45$ mM could be extracted.

*Steady-State Velocities To Determine K_i**. The enzyme (9.1 nM) was incubated for 24 h with 10 nM to 10 μM L-Ala-P. Activity was then assayed in the L to D direction at [S] $\ll K_m$ with the coupled assay, and the data were plotted according to the Henderson (1972) method $[I_t]/(1 - v/v_c)$ vs. v_c/v, where v_c is the velocity of the control without L-Ala-P; the line obtained (Figure 3) has a slope $K_i^* = 1.25$ μM, in good agreement with the value of 1.2 μM from the Lineweaver–Burk enzymes at steady state; this slope remained unaffected by [S].

Recovery of Catalytic Activity after Preincubation with L-Ala-P. The final set of kinetic studies on the inactivation dealt with regain of catalytic activity after preincubation of enzyme with L-Ala-P. We noted above that negligible regain of activity occurred on intermediate assay after dilution of enzyme from preincubation with inhibitor; however, a very slow release process could be monitored over several hours to days. Thus, Figure 4a displays regain of activity in enzyme that had been inactivated for 30 min with [^{14}C]-L-Ala-P (pH 7.4) and then subjected to gel filtration chromatography to remove excess inhibitor. The inactive protein, containing ^{14}C label stoichiometric with the amount of bound PLP coenzyme, was then diluted 500-fold in the same buffer (to a final concentration of 19 μM) and the regain of catalytic activity followed during the subsequent 220-h period in both the L-Ala to D-Ala (CHES or HEPES buffer, pH 9 or pH 7.4, respectively) and the D-Ala to L-Ala direction (CHES buffer, pH 9) with 10

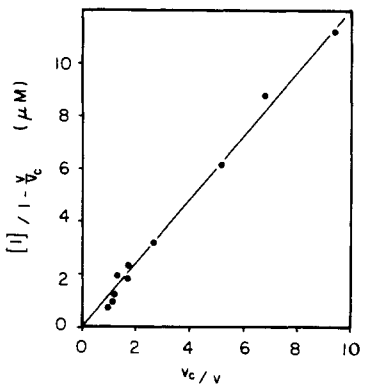


FIGURE 3: Henderson plots for inhibition of *B. stearothermophilus* alanine racemase by L-Ala-P. The enzyme (9.1 nM) was preincubated for 24 h in 100 mM HEPES, pH 7.4, at 37 °C with various amounts of L-Ala-P (0, 0.075, 0.1, 0.2, 0.5, 0.75, 1, 2, 5, 7.5, and 10 μM). Activity was checked in the same buffer at 0.5 mM L-alanine (well below K_m); in these conditions, the observed initial velocity approximates the steady-state velocity (if it was not reached) and the slope of the plot equals K_i^*. v and v_c are velocities in the presence and absence, respectively, of inhibitor.

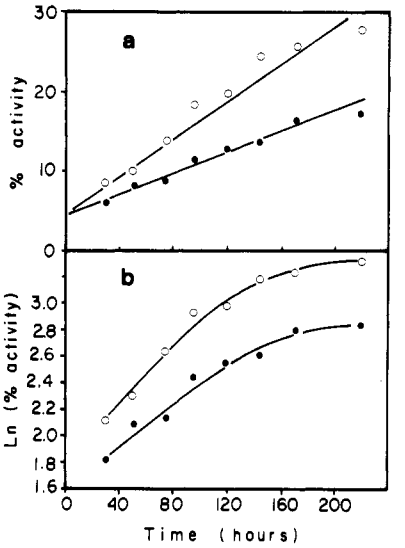


FIGURE 4: Reversal of L-Ala-P inhibition of *B. stearothermophilus* alanine racemase upon dilution. A total of 1.79 mg (22 nmol) of enzyme was inactivated in 1 mL of 100 mM HEPES, pH 7.4, with 1.2 mM [U-^{14}C]-L-Ala-P. The enzyme inactivated to 96% was submitted to gel filtration; the inactive protein was then diluted 500-fold in 100 mM HEPES, pH 7.4 (19 μM final), and the regain of activity followed in L to D (CHES or HEPES, pH 9 or 7.4) and in L to D (CHES, pH 9) directions as described under Assays with 10 μL of the diluted solution. (a) Percent activity vs. time. (b) In (% activity) vs. time.

μL of the above stock enzyme solution in a 1-mL assay cuvette (final concentration in assay mixture 380 nM).

As shown in Figure 4a, there is measurable zero-order regain of activity as assayed in either direction. For the L to D assay, the activity goes from 7% of initial to 28% of a control sample (treated the same way without inhibitor) on standing of this thermophilic enzyme–L-Ala-P complex for 220 h at room temperature. The data of Figure 4a are replotted in a log activity regain vs. time analysis in Figure 4b. This plot shows the data deviate from linearity, but the curves were fitted by linear regression to a first-order process. From the extrapolation in Figure 4a, the $t_{1/2}$ values of ca. 770 or 420 h (L to D or D to L assay, respectively) were estimated. If the process of activity regain is treated as a first-order process in Figure 4b, the k for activity regain would be 10^{-4} min^{-1} for each line and correspond to a $t_{1/2}$ of 115 h. This latter analysis

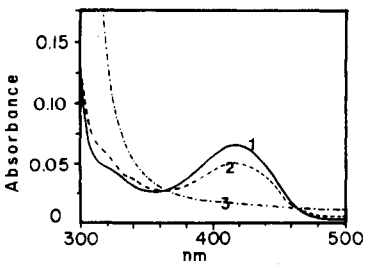


FIGURE 5: UV–visible spectra of *B. stearothermophilus* alanine racemase. (1) Racemase after inactivation with [U-^{14}C]-L-Ala-P and separation of the labeled protein from small molecules on Sephadex G-25. (2) After treatment of the above solution with solid $NaBH_4$. (3) After simultaneous addition of $NaBH_4$ and 5 M KOH (20 μL).

is obviously inappropriate since in 115 h one in fact sees only a net 12% regain of activity relative to the control. These measurements are obviously difficult due to the long times involved. We tentatively assign an average $t_{1/2}$ of 600 h [(770 + 420)/2], corresponding to a net dissociation of intact L-Ala-P from *B. stearothermophilus* alanine racemase once every 25 days. Even if this value were off by a factor of 2 this is a dramatically long-lived enzyme–inhibitor noncovalent complex. We note in passing that Cha (Cha et al., 1975) in his study on regain of catalytic activity of adenosine deaminase from preincubation with coformycin also observed apparent zero-order regain, even though $t_{1/2}$ is minutes rather than days as in our case.

Studies on Nature of Binding of Ala-P to B. stearothermophilus Alanine Racemase. During treatment of 1.79 mg of pure enzyme (22 μM enzyme) with 1.2 mM [^{14}C]-L-Ala-P for 30 min to a final loss of 96% of the initial activity, there was essentially no change in the PLP chromophore associated with the enzyme. The A_{280}/A_{420} ratio of native enzyme was 7.54; in the inactive enzyme it was 7.94. Analysis of radiolabel associated with the protein by subsequent gel filtration confirmed radioactivity stoichiometric with bound PLP (e.g., 0.55 mol/mol of enzyme subunit, vide supra). During inactivation, there is no detectable C–H cleavage in [2-^{3}H-DL-Ala-P, and the question arises whether Ala-P isomers actually form an aldimine linkage, displacing the active site lysine $-NH_2$ group.

Attempts to trap an Ala-P–PLP aldimine by BH_4^- reduction were unsuccessful here as they had been in our recent studies with the *Sr. faecalis*, where much less protein had been available. Figure 5 shows the UV–visible spectrum of [^{14}C]-DL-Ala-P-inactivated racemase in curve 1. Treatment with a large excess of $NaBH_4$ (enough to reduce the native enzyme's PLP chromophore instantly) had only slight effect over many minutes as exemplified in curve 2. Again, in contrast an Ala–PLP–enzyme complex was reduced in seconds. Only when one denatures the [^{14}C]Ala-P-inactivated enzyme can borohydride gain access to the PLP chromophore as shown in curve 3 produced by simultaneous addition of BH_4^- in 5 M KOH. To ascertain whether any PNP–Ala-P had been trapped, the supernatant was neutralized and chromatographed on HPLC. Radioactivity comigrated with free Ala-P but not with authentic PNP–Ala-P made by reduction of Ala-P and PLP free in solution. The efficiency of this aldimine trapping process was tested with [^{14}C]-L-alanine–borohydride reduction of a mix consisting of alanine racemase (50 nmol) and [^{14}C]-L-Ala (40 mM) in the inactivation conditions followed by gel filtration, and HPLC afforded the reduced aldimine adduct PNP–Ala in a 10% overall yield, so enzyme and normal substrate give a low but detectable yield for BH_4^- reduction.

Modification of Bound PLP Fluorescence Spectrum. Although BH_4^- failed to give any evidence that L-Ala-P was in combination with the PLP coenzyme in inhibited enzyme,

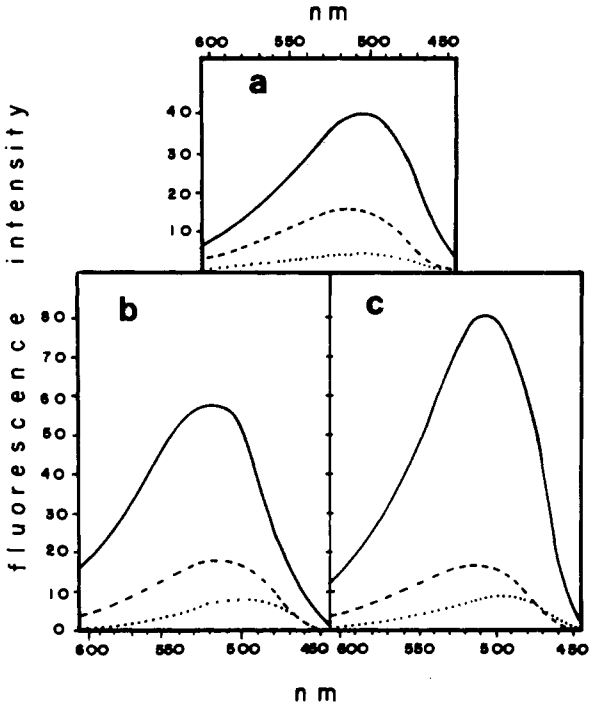


FIGURE 6: Fluorescence emission spectra of *B. stearothermophilus* alanine racemase (1 mg/ml) after incubation with (a) D- or L-alanine (50 mM), (b) L-Ala-P (1 mM), or (c) D-Ala-P (1 mM) for 20 min at 37 °C. (—) Complete mixture; (– –) native enzyme alone; (···) without enzyme + 20 μM PLP. Excitation was at 420 nm.

fluorescence of the racemase-bound B_6 coenzyme can be used as a qualitative measure of the microenvironment around the PLP, by excitation at 420 nm and monitoring the emission in the 440–600-nm region. With 1 mg of enzyme in 1 mL of HEPES buffer, pH 7.4, D- or L-alanine (50 mM) had the perturbing effect noted in Figure 6a, where either enantiomer of substrate produced an increased emission peak centered around 505 nm with a relative F value of 40. As depicted in Figure 6b for 1 mM L-Ala-P and in Figure 6c for 1 mM D-Ala-P, each slow-binding enantiomer generated a fluorescence increase at 505–515 nm but of higher and differential relative fluorescence units. Thus, 1 mM L-Ala-P gave F_{515} = 57.5 and 1 mM D-Ala-P gave F_{505} = 80, consistent with binding in the asymmetric microenvironment of enzyme-attached PLP and the lack of interconversion of Ala-P isomers by the enzyme.

Effect of Ala-P on CD Spectrum. To probe further the interaction of Ala-P with the bound PLP coenzyme, CD spectra were recorded for both native *B. stearothermophilus* alanine racemase (1 mg/mL) (curve 1, Figure 7) and the same enzyme after inactivation with 1 mM L-Ala-P for 12 h (curve 2, Figure 7). There is a striking decrease in the negative CD absorbance centered around 420 nm in the native enzyme. The loss of PLP-associated CD bands has been detected previously, for example, with L-aspartate transaminase (Martinez-Carrion et al., 1979), where the decrease accompanies transaldimination, and was consistent with formation of a substrate–PLP aldimine or an inhibitor–PLP aldimine. An equivalent decrease of CD absorbance at 420 nm occurs on binding substrate alanine in mesophilic alanine racemases (N. Galakatos and C. Walsh, unpublished data). Similarly, we suggest the CD loss at 420 nm on Ala-P time-dependent inhibition is an accumulation of an Ala-P–PLP aldimine complex (no absorption change at 420 nm) in the EI* long-lived complex.

Active Site Peptide Structure of B. stearothermophilus Alanine Racemase. We have begun to elucidate the structural

Table II: Sequence Homology of Racemase Active Site[a]

Sl. typhimurium	
dadB	Val-Trp-Ser-Val-Val-Lys*-Ala-Asn-Ala-Tyr-Gly-His-Gly-Ile-Glu-Arg
alr	Leu-Val-Ala-Val-Val-Lys*-Ala-Asn-Ala-Tyr-Gly-His-Gly-Leu-Leu-Gln-Thr
B. stearothermophilus	Ala-Pro-Pro-Lys*-Ala-Asn-Ala-Tyr

[a] Residues marked with an asterisk (*) carry the radioactivity from $B^3H_4^-$ reduction of E-PLP.

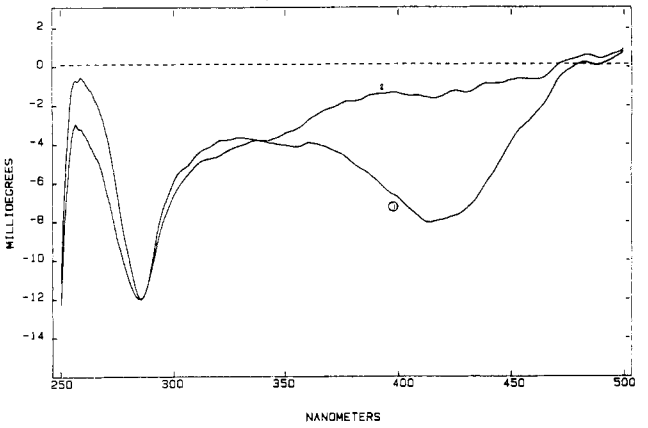


FIGURE 7: Circular dichroism spectrum of *B. stearothermophilus* alanine racemase (1 mg/mL HEPES, pH 7.4): (curve 1) native enzyme; (curve 2) enzyme 12 h after addition of 1 mM L-Al-P at 4 °C. CD spectra were recorded on an AVIV Spectropolarimeter Model 60 DS, in 1-mm cells at 4 °C.

basis for time-dependent inhibition by Ala-P of three alanine racemases from Gram-positive bacteria. No such slow-binding phenomena occur with alanine racemases from Gram-negative bacteria. We have determined the partial sequence of the active site tryptic peptide from *B. stearothermophilus* racemase and compare it here to the two sequences we have recently obtained for two alanine racemases cloned from *S. typhimurium* (Badet & Walsh, 1984; Esaki & Walsh, 1986). The usual procedure of $B^3H_4^-$ reduction of native enzyme (5 mg) to generate [^{3}H]PNP–NH_2-Lys–enzyme was followed by reductive alkylation and tryptic digestion, HPLC isolation of radiolabeled peptides, and automated Edman degradation for sequence determination (Badet et al., 1984). Seven residues around the [^{3}H]PNP–Lys adduct could be unambiguously determined and are shown in Table II in comparison with the *dadB* and *alr* gene-encoded *Salmonella* racemases. Although we have been unable to identify the residue of cycle B of this octapeptide, the sequence homology between the Gram-negative and Gram-positive racemases as yet gives no clue to the irreversible behavior of Ala-P as time-dependent inactivator selective for the latter class.

Analogues of Ala-P as Time-Dependent Inhibitors. In our initial study on the *Sr. faecalis* alanine racemase, we recently found that the dimethyl analogue of Ala-P but not either D or L isomer of Ala–phosphinate was a time-dependent inactivator and that correlation holds with the *B. stearothermophilus* alanine racemase. In Table III, we now note that the substitution of the methyl group in Ala-P by fluoromethyl or monochloromethyl retains inactivating properties but neither the more bulky dichloromethyl- nor trichloromethyl-Ala-P inactivates. To test whether the phosphonate dianion was crucial, the monomethyl and dimethyl esters of Ala-P were tested. The Ala-P dimethyl ester was clearly inert, but the monomethyl ester showed slow onset of inactivation at high (20 mM) concentrations. We estimate that a 1% contamination of the hydrolysis product (possibly generated in stiu during the incubation period) would account for the phenomenon and at present suggest the free acid is the inactivator in those solutions.

Table III: Inhibition Parameters of Phosphono Amino Acid Analogues for *B. stearothermophilus* Alanine Racemase

compd	K_i (mM)	K_i* (μM)
L-Ala-P	1	1.25
β-fluoro-Ala-P		1.4
β-chloro-Ala-P	1.2	28.5
β-dichloro-Ala-P	*a*	*a*
β-trichloro-Ala-P	*a*	*a*

[a] No detectable inhibition.

Discussion

The phosphonic acid analogue of L-alanine (L-Ala-P) is an effective antibacterial agent when presented outside the cell as the L-Ala-L-Ala-P dipeptide, transported by the dipeptide permease, and hydrolyzed enzymically inside the bacterial cell to L-Ala and L-Ala-P, which has as its target alanine racemase. The results presented in this paper add the pure alanine racemase from *B. stearothermophilus* to our recently reported cases of *S. faecalis* (Badet & Walsh, 1984) and *Staphylococcus aureus* (Roise, 1984) racemases, where these three racemases from Gram-positive bacteria are inactivated in time-dependent fashion by L-Ala-P or D-Ala-P. In contrast, we find alanine racemases we have purified from four Gram-negatives, *E. coli* B (Wang & Walsh, 1978), *Pseudomonas striata* (Roise et al., 1984), and *Sl. typhimurium* (both *dadB*- and *alr*-encoded racemases; Badet et al., 1984; Esaki & Walsh, 1986), are not susceptible to time-dependent inhibition.

The thermophilic *B. stearothermophilus* racemase appears to be the catalyst of choice for analysis of the Ala-P time-dependent inactivation process. The enzyme, cloned and overproduced in *E. coli*, is now available in quantity, which contrasts with the tedious purification of the *Sr. faecalis* racemase which we had to purify 30 000-fold to obtain 0.1 mg of protein (Badet & Walsh, 1985).

As in the *Sr. faecalis* case, the *B. stearothermophilus* racemase shows no capacity to racemize Ala-P or even to carry out C–H cleavage. Inactivation involves no obvious chemical transformation of Ala-P but only a set of slowly reversible binding equilibria between intact Ala-P and enzyme. We have been able to characterize several kinetic features of the "slow-binding" (Morrison, 1982) inhibition and observe the switch over from initial competitive inhibition toward substrate alanine to apparent noncompetitive inhibition at some later time *t*. This noncompetitive behavior reflects a lessened amount of active enzyme in solution since with increasing Ala-P, an increased mole fraction of enzyme accumulates as E···Ala-P and does not noticeably dissociate in subsequent initial velocities assays.

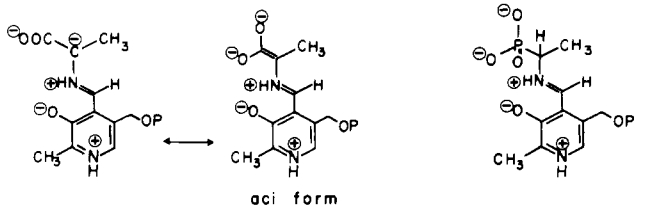
The initial K_i is ca. 1 mM by several methods of estimation while the K_i* in the later "noncompetitive" assays is reduced 3 orders of magnitude to ca. 1 μM. By these criteria, Ala-P isomers are not tight-binding inhibitors in contrast with methotrexate (Williams et al., 1979) or coformycin (Cha et al., 1975), which have initial K_i values in the nanomolar range for their enzyme targets, dihydrofolate reductase and adenosine deaminase, respectively. The onset of irreversible inhibition is slow; at saturating Ala-P concentration, an extrapolated value of 10 min^{-1} is obtained, and so, Morrison's category of

slow-binding inhibitor is applicable. Typically in such cases, formation of the initial EI complex is rapid, with bimolecular rate constants of 10^5–10^7 M^{-1} s^{-1} in the range of normal rate constants for ES formation (Fersht, 1984), but in our case, the net "k_{on}" detected is a paltry 6–8 M^{-1} s^{-1}. This value is much too low to represent an initial collisional complex formation and must reflect multiple elementary steps that precede a first-order EI isomerization that is functionally irreversible.

If the onset of time-dependent loss of activity is moderately slow (10 min^{-1}) the regain of activity by net dissociation of intact Ala-P from an inhibited enzyme complex is dramatically difficult to measure accurately. An estimated $t_{1/2}$ of 600 h (25 days) is remarkable. First, this is considerably longer than the lifetime of all other slow-binding inhibitors yet measured except the carboxyarabinitol bisphosphates to ribulosebisphosphate carboxylase, where Pierce et al. (1980) estimated ca. 15–17 days and Schloss and Lorimer (1982) with a single inhibitory diastereoisomer suggested some 5–6-day half-life. With that exception, other dissociations are in the minutes to hours range. We think it more appropriate to think of this class of inhibitors not so much as slow binders but as slow dissociators (Anderson et al., 1984), and this is particularly valid for the Ala-P–Gram-positive alanine racemase complexes.

Without having the individual rate constants for the inhibition process, we cannot be quantitative about the equilibria favoring isomerization of an initial racemase–Ala-P complex to an isomerized one, but one can estimate 600 h/0.07 min or about $5 \times 10^5/1$ against return to an enzyme form from which Ala-P can dissociate.

Key questions are why and how Ala-P and congeners are slow-dissociating inhibitors. In terms of peculiarities of Gram-positive racemases, our initial active site tryptic octapeptide sequence information shows homology with the *Salmonella alr* gene-encoded racemase (Esaki & Walsh, 1986), and the latter Gram-negative racemase is unsusceptible. Therefore, it is quite likely one will need X-ray structures of native Gram-negative and Gram-positive alanine racemases and of the latter cocrystallized with D- or L-Ala-P to see whether any structural clues emerge at that level of analysis. We were uncertain whether Ala-P forms an aldimine with the PLP coenzyme since BH_4^- fails to gain access to the tight complex and so can capture neither resting lysyl–PLP aldimine or Ala-P–PLP aldimine. One of the two must be present given the optical spectrum of inhibited enzyme, but the isomerization leading to the complex with the slow dissociation rate must block access of BH_4^- to the active site. Fluorescence perturbation does suggest D- or L-Ala-P is in the vicinity of the coenzyme in the accumulated inactive enzyme complex. Circular dichroism studies on the ALA-P inhibited enzyme point strongly to transaldimination and accumulation of an Ala-P–PLP Schiff base on the E″I* complex. If Ala-P is in aldimine linkage to PLP in the isomerized complex, reversal may be slow because water is excluded from the active site or because the Ala-P dianion is a mimic of the aci form of the substrate–PLP carbanion presumed to be an intermediate in normal catalysis:



The precedents for nitronate anions as transition-state analogues for aci forms of carbanions have accumulated in recent years, and the aconitase case is notable where the nitronate anion analogue of isocitrate with a $K_i = 10^{-10}$ M binds 10^5-fold more tightly than isocitrate itself ($K_m = 10^{-5}$ M) (Schloss et al., 1982). The phosphonate dianion appears crucial for the time-dependent inhibition, and this would be consistent with such an hypothesis, albeit the phosphonate dianion is not planar like the substrate–PLP carbanion or nitronate analogues. Initial studies on β-halo-Ala-P species were to see if one could combine β-haloalanine-type irreversible inactivation, proceeding by HX elimination and covalent capture of the PLP (Badet et al., 1984; Roise et al., 1984), with the slow dissociation mode. Only the monohalo substituent is tolerated in the chloro series, and there is no evidence for the HX elimination there. Thus, the combination of the two time-dependent inactivation modes may not be readily realized unless racemases can carry out C–H cleavage on amino phosphonate analogues.

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Registry No. L-Ala-P, 60687-36-7; D-Ala-P, 66068-76-6; 3-fluoro-Ala-P, 101493-65-6; 3-chloro-Ala-P, 70350-66-2; dimethyl *N*-carbobenzyloxy(1-aminoethyl)phosphonate, 82629-22-9; diphenyl *N*-carbobenzyloxy(1-aminoethyl)phosphonate, 65164-80-9; monomethyl (1-aminoethyl)phosphonate, 61937-80-2; dimethyl (1-aminoethyl)phosphonate, 77526-64-8; alanine racemase, 9024-06-0.

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Preparation and Some Properties of 6-Substituted Flavins as Active Site Probes for Flavin Enzymes†

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ABSTRACT: 6-Azidoflavins, 6-thiocyanatoflavins, and 6-mercaptoflavins at the lumiflavin, riboflavin, FMN, and FAD level were prepared from the corresponding 6-aminoflavins and some of their properties investigated. They are bound tightly by apoflavin enzymes which bind either riboflavin, FMN, or FAD. 6-Azidoflavins undergo facile photolysis. One major product was identified as 6-aminoflavin. A further product, which was formed also during acid decomposition of the azide, results from opening of the flavin benzene ring and is proposed to have a lumazine structure. 6-Thiocyanatoflavins are easily converted by dithiothreitol to 6-mercaptoflavins. The latter are stabilized against dimerization in the presence of reducing thiols. 6-Mercaptoflavins have a pK of 5.9, which corresponds to ionization of the 6-SH function. The neutral form is yellow, while the anion is green, due to a long-wavelength band (λ_{max} ~600 nm) extending beyond 700 nm. These properties suggest the use of these 6-substituted flavins for probing the active site of flavin enzymes. Because their reactive substituents are in close proximity to the flavin N(5)-position, these 6-substituted derivatives should also serve as useful probes of the environment around the flavin N(5), a position known to be involved in all flavin-mediated redox processes.

Our present understanding on the ways flavins interact with the protein at the active center of enzymes has resulted to a considerable extent from the use of flavin-derived active site probes (Ghisla et al., 1976; Walsh et al., 1978; Massey et al., 1979, 1984; Schopfer et al., 1981; Biemann et al., 1983; Kasai et al., 1983; Ghisla et al., 1984; Fitzpatrick et al., 1985; Krauth-Siegel et al., 1985). They might be differentiated into three broad classes with respect to their chemical properties: (i) flavin analogues that reflect active site properties by virtue of spectral effects induced upon binding; (ii) probes that react either with a protein functional group at the active site or with solvent-borne reactants (accessibility probes); (iii) photoaffinity labeling derivatives. Until the present, functions at positions 1, 2, 4, and 8 of the flavin molecule have been candidates for specific modifications, yielding information, respectively, about the interactions with the protein or solvent of positions in all three rings of the flavin structure. One part of the flavin, which has been little explored in this context, is the portion of the benzene ring closest to the catalytically important N(5) position. Exceptions have been the use of isoflavin (6-methyl-8-norisoalloxazine) (Choong & Massey, 1981; Hastings et al., 1981) and of 6-hydroxyflavin coenzyme analogues, the latter having proven useful in the study of pK shifts induced upon binding, by virtue of their pK ~ 7 (Mayhew et al., 1974; Thorpe & Massey, 1983). We thus set out to attempt to introduce two types of functional groups into this part of the molecule. The azido function should serve as a photoaffinity label (Bayley & Knowles, 1977; Chowdhry & Westheimer, 1979; Lwowski, 1980), as has proven feasible in the case of 8-azidoflavins (Ghisla et al., 1984; Fitzpatrick et al., 1985). A mercapto function might serve as a probe for investigating the accessibility to this region of the flavin, by using its readily monitored reactivity with alkylating agents. In addition, it could also function as a chromophore sensitive to changes in the dipole moment of the active center, since it is expected to be comparable to 6-hydroxy- and 8-hydroxyflavins (Mayhew et al., 1974; Ghisla & Mayhew, 1976) and to 8-mercaptoflavins (Massey et al., 1979) and to have a pK in the physiological range. The synthesis and properties of some 6-substituted flavins have been described earlier in the context of the structural elucidation of 6-*S*-cysteinylriboflavin, the co-

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