^{13}C NMR Studies of the Enzyme–Product Complex of *Bacillus subtilis* Chorismate Mutase†

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ABSTRACT: The chorismate mutase reaction is a rare enzyme-catalyzed 3,3-sigmatropic rearrangement of chorismate to prephenate. *Bacillus subtilis* chorismate mutase was overproduced and purified from *Escherichia coli* XL1-Blue (pBSCM2) using a modification of the procedure of Gray et al. (Gray, J. V., Grolinelli-Pimpaneau, B., & Knowles, J. R. (1990) *Biochemistry 29*, 376–383); the modification leads to minimal contaminating prephenate dehydratase activity (<0.001%). The native molecular mass of *B. subtilis* chorismate mutase was determined by gel filtration to be ~44 kDa, indicative of a homotrimer of the 14.5-kDa subunits as determined by electrospray mass spectrometry. ^{13}C NMR was used to study the structure of [U-^{13}C]prephenate bound at the active site of *B. subtilis* chorismate mutase. All the enzyme-bound ^{13}C NMR resonances of [U-^{13}C]prephenate were assigned, and where possible, $^{1}J_{C,C}$s were quantified; [1,3,5,8-^{13}C]prephenate and [2,6,9-^{13}C]prephenate, prepared respectively from [1,3,5,8-^{13}C]-chorismate and [2,6,9-^{13}C]chorismate, aided the ^{13}C NMR resonance assignments. Enzyme-bound prephenate exhibits remarkably different chemical shifts relative to free prephenate; the chemical shift changes range from –6.6 ppm for the C_6 resonance to 5.6 ppm for the C_5 resonance, suggesting a strong perturbation of the C_5–C_6 bond. ^{13}C NMR studies of model compounds at various pH values and in various solvents suggest that the observed ^{13}C chemical shift changes of enzyme-bound prephenate cannot be rationalized solely on the basis of changes in the pK_as of the carboxylic acid groups or hydrophobic solvation at the active site. With regard to the chemical mechanism of the chorismate mutase-catalyzed reaction, these NMR studies do not provide any evidence for a dissociative mechanism which involves discrete intermediates.

Chorismate is the branch-point intermediate necessary for the biosynthesis of the amino acids tyrosine, phenylalanine, and tryptophan (Gibson & Gibson, 1964; Edwards & Jackman, 1965). The conversion of chorismate to prephenate, the first committed step in the biosynthesis of tyrosine and phenylalanine, is illustrated in Figure 1; this reaction, catalyzed by chorismate mutase (CM, E.C. 5.4.99.5), is a rare enzyme-catalyzed 3,3-sigmatropic rearrangement. CM occurs in plants and microorganisms (Ganem, 1978; Poulsen & Verpoorte, 1991) and is not found in animals. Design of efficient nontoxic inhibitors of CM, which might serve as antibiotics or herbicides, requires an understanding of the reaction mechanism and the structures of the ligands bound at the active site.

The mechanism of the CM reaction has been under investigation for at least two decades, and there is still no consensus. Several mechanisms have been proposed for the CM-catalyzed reaction or for the analogous thermal reaction based on kinetic studies (Andrews et al., 1973; Copley & Knowles, 1985), molecular orbital calculations (Andrews et al., 1973, 1977; Andrews & Haddon, 1979), isotope effects (Addadi et al., 1983; Guilford et al., 1987; Delany et al., 1992), and the reactivities of substrate analogues (Gajewski et al., 1987; Pawlak & Berchtold, 1988; Pawlak et al., 1989) or transition-state analogues (Bartlett et al., 1988; Clarke et al., 1987, 1990). Most available data support a mechanism where C_3–O bond breakage precedes C_9–C_1 bond formation,

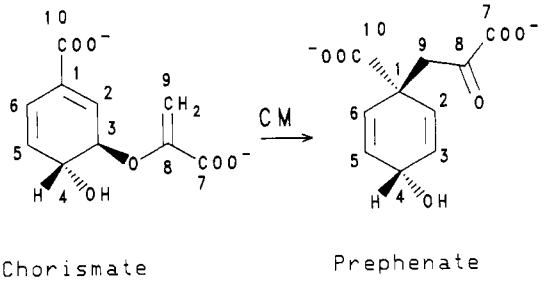

FIGURE 1: The CM-catalyzed reaction.

either through an asynchronous concerted rearrangement or through a dissociative mechanism involving the formation of discrete intermediates; it has also been proposed that CM preferentially binds the less abundant pseudodiaxial conformer of chorismate to facilitate the reaction (Copley & Knowles, 1987; Gray et al., 1990a).

^{13}C NMR is a powerful probe of enzyme-catalyzed reactions, like that of CM, which involve formation or breakage of bonds to carbon. The method allows one to observe the effects of enzyme binding on reaction components and may allow the direct observation of reaction intermediates (Allerhand, 1979; Jaffe & Markham, 1987; Barlow et al., 1989). The difference in ^{13}C NMR chemical shifts between free and bound species is a direct measure of changes in electron density at ^{13}C-labeled carbons and allows inference as to where enzyme binding exerts changes in protonation states, tautomeric forms, charge distribution, and/or molecular geometry. To facilitate the ^{13}C NMR investigation of the CM reaction mechanism, we have recently prepared and assigned the ^{13}C NMR spectra of [U-^{13}C]chorismate and [U-^{13}C]prephenate (Rajagopalan et al., 1992). The use of U-^{13}C ligands provides measurable

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carbon–carbon coupling constants ($J_{C,C}$) as an additional molecular probe; the $J_{C,C}$s of [U-^{13}C]chorismate and [U-^{13}C]-prephenate have also been quantified (Rajagopalan et al., 1992). The molecular mass, availability, and bifunctional nature of the *Escherichia coli* CMs limited ^{13}C NMR studies of CM in the past. These issues were apparently resolved when Gray et al. (1990b) overproduced the monofunctional *Bacillus subtilis* CM in *E. coli* to 30–35% of the soluble cell protein and reported it to be the smallest of the natural CMs known so far, a homodimer of 14.5-kDa subunits. Gray et al. (1990a) reported the ^{13}C NMR chemical shifts of [7,8,9-^{13}C]prephenate bound to *B. subtilis* CM, yet provided little interpretation of the changes in chemical shifts. That study dealt solely with the pyruvoyl side chain of prephenate. In this study we include all the carbons of prephenate, quantify coupling constants for enzyme-bound species, and include model compounds to assist in the interpretation of chemical shifts. Another prior NMR study of the CM-catalyzed reaction looked for the accumulation of intermediates in a multiturnover ^{1}H NMR experiment which monitored the yeast CM-catalyzed reaction (Schmidheini et al., 1990); none were observed.

The size of a protein has a profound effect on the line widths of methylene or methine ^{13}C NMR resonances and determines whether a particular carbon needs to be deuterated in order to observe its ^{13}C NMR resonance in a protein complex (Jaffe & Markham, 1988; Malthouse & Finucane, 1991). Therefore, in this paper, we have redetermined the size of *B. subtilis* CM and found it to be a trimer of ~44 kDa. This size is larger than previously reported (Gray et al., 1990b) but still small enough to allow observation of relatively sharp resonances for ^{13}C centers with directly attached protons. For proteins of <80-kDa size the calculated line widths of methine ^{13}C NMR resonances (<35 Hz) are within the limits of detection on a 7T spectrometer (Jaffe & Markham, 1987). Although deuteration of the methylene or methine carbons would result in sharper enzyme-bound ^{13}C NMR resonances, it is not essential for ligands bound to *B. subtilis* CM. Most significantly, we have observed and completely assigned the ^{13}C NMR spectrum of [U-^{13}C]prephenate bound at the active site of *B. subtilis* CM. There are remarkable changes in chemical shifts and coupling constants when prephenate binds to the enzyme, which indicate that the CM active site environment is both asymmetric and quite dissimilar from aqueous solution.

EXPERIMENTAL PROCEDURES

Materials. DIFCO yeast extract and tryptone were obtained from VWR Scientific, Bridgeport, NJ. Antibiotics, isopropylthiogalactoside (IPTG), D_2O, acetone-d_6, DMSO-d_6, $CHCl_3$-d, pyruvic acid, dithioerythritol (DTE), phenylmethanesulfonyl fluoride (PMSF), glycerol, and phenylalanine were purchased from Sigma Chemical Co., St. Louis, MO. Cell Debris Remover (CDR) and DE-52 cellulose are products of Whatman Laboratories, Clifton, NJ. Sephacryl S-100 and Superdex 70 were purchased from Pharmacia, Piscataway, NJ. Zorbax GF-450 was obtained from DuPont. Molecular weight standards were obtained from Pharmacia and Bio-Rad, Richmond, CA. The bacterial strain Kp 62-1 was obtained from The American Type Culture Collection, Rockville, MD. Chorismate, [U-^{13}C]chorismate (>98% ^{13}C), [1,3,5,8-^{13}C]chorismate (50%, 14%, 32%, and 46% ^{13}C at C_1, C_3, C_5, and C_8, respectively), and [2,6,9-^{13}C]chorismate (36%, 24%, and 34% ^{13}C at C_2, C_6, and C_9, respectively) were prepared from glucose, [U-^{13}C]glucose (98.9%), [2-^{13}C]-glucose (99%), and [1-^{13}C]glucose (99%), respectively, using the culture filtrates of Kp 62-1 as described elsewhere (Rajagopalan et al., 1992; Rajagopalan & Jaffe, 1993). ^{13}C-Labeled prephenate was prepared from purified ^{13}C-labeled chorismate in 50 mM potassium phosphate (KPi) at pH 8.0 using a catalytic amount of *B. subtilis* CM. Protein concentration was estimated using Pierce Coomassie protein assay reagent obtained from Pierce, Rockford, IL, and bovine serum albumin was used as the calibration standard. All other chemicals were reagent grade.

Overproduction and Purification of B. subtilis CM. The plasmid pBSCM2 was a gift from Professor J. R. Knowles, Harvard University; it was transformed into *E. coli* XL1-Blue (pBSCM2) according to Gray et al. (1990b). Luria broth (LB) plates containing ampicillin (200 μg/mL) and tetracycline (20 μg/mL) were streaked with a glycerol freeze of *E. coli* XL1-Blue (pBSCM2). Single colonies were picked and grown in 50 mL of LB medium at 37 °C supplemented with the above antibiotics to yield an overnight starter culture; this was used to inoculate a 1-L growth. The production of *B. subtilis* CM was induced at an OD_{550nm} of 1.0 by the addition of IPTG (5 mM). The cells were grown overnight and harvested by centrifugation, yielding 7.5 g of cells/L. The cells were flash-frozen and stored at −70 °C.

The cells were processed according to Gray et al. (1990b), but the purification procedure has been modified to minimize the contaminating prephenate dehydratase (PD) activity as described below. Fifteen grams of cells was suspended in 150 mL of buffer A (50 mM KPi at pH 7.6, 1 mM DTE, 1 mM PMSF, 5% (v/v) 2-propanol, and 10% (v/v) glycerol), and the cells were lysed using a French press at 20 000–22 000 psi. The cell debris was removed by centrifuging the suspension at 10000*g* for 30 min. The supernatant was diluted to 300 mL with buffer A and stirred with 5 g of CDR at 4 °C for 5 min. The resulting slurry was vacuum-filtered through 41 g of CDR which had been preequilibrated with buffer A; the CDR is not re-used. The filtrate containing partially purified *B. subtilis* CM was loaded onto a 5 × 30 cm DE-52 cellulose column; most of the contaminating proteins remained on the column, while *B. subtilis* CM was eluted with buffer A. Fraction collection (10 mL/fraction) began immediately, and CM activity first appeared at fraction 44. The fractions containing CM activity were pooled and concentrated down to 20 mL using a pressure cell with an Amicon PM-10 membrane; NaCl solution (2 M) was added to the concentrated protein solution to a final concentration of 0.2 M. The concentrate was loaded onto a 5 × 91 cm Sephacryl S-100 column equilibrated with buffer A containing 0.2 M NaCl and eluted at 1.5 mL/min. *B. subtilis* CM elutes between 842 and 931 mL. The pooled CM activity was concentrated by ultrafiltration as above.

Enzyme Assays. CM was assayed at 30 °C by measuring the rate of disappearance of chorismate at 273 nm ($\Delta\epsilon_{273nm}$ = 2.63 mM^{-1} cm^{-1}) in 1.2 mL of assay buffer containing 50 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 10 mM 2-mercaptoethanol, and 0.5 mM chorismate (Turnbull et al., 1991). The reaction was started by the addition of enzyme (~70 ng). The contaminating PD activity was measured at 30 °C by monitoring the production of phenylpyruvate from 0.5 mM chorismate in 1.0 mL of the above assay buffer, containing 35 μg of total protein; aliquots (225 μL) were removed at 15-min intervals and mixed with 25 μL of 11 N NaOH, and the absorbance at 320 nm was measured (ϵ_{320nm} = 17.6 mM^{-1} cm^{-1}) (Gething et al., 1976).

NMR Studies. Protein samples for ^{13}C NMR studies were dialyzed against 50 mM KPi and concentrated using a Centricon-10 apparatus (Amicon) which had been pretreated with bovine serum albumin (10 mg/mL) to block any nonspecific protein binding sites. D_2O was added to a concentration of 15%, and where noted, L-phenylalanine was added to a concentration of 3 mM. The [^{1}H]^{13}C NMR spectra were run on a Bruker AM-300 spectrometer equipped with multinuclear 10- and 5-mm probes. These spectra were accumulated in blocks of 2000 scans at 25 °C, using a spectral width of 19 230.7 Hz, 32K data points, and a pulse width of 4.5 μs (45°), with composite pulse decoupling using a 3.8-s recycle time. The NMR spectra were processed using the program Felix 2.0 (Hare Research Inc.). [^{1}H]^{13}C NMR spectra were also obtained on a Bruker AM-600 spectrometer at 25 °C with composite pulse decoupling, using the following parameters: spectral width, 38 461.5 Hz; pulse width, 7.0 μs (45°); recycle time, 3.4 sec; and 32K data points. ^{13}C NMR chemical shifts are referenced to dioxane as external standard at 67.4 ppm.

The pH titration of phenylpyruvate used 3.5 mM [U-^{13}C]-phenylpyruvate in 50 mM KPi buffer with 15% D_2O. [U-^{13}C]-Phenylpyruvate was prepared from [U-^{13}C]prephenate in an acid-catalyzed decarboxylation reaction by lowering the pH to 4.5. The [^{1}H]^{13}C NMR spectra of [U-^{13}C]phenylpyruvate were acquired with 400–800 scans, composite pulse decoupling, and a 3.8-s recycle time. Pyruvic acid (300 mM) in 2.5 mL of 50 mM KPi (15% D_2O) was used for the pH titration using the same parameters as above; only 64 scans were required. For all pH titrations the pH was adjusted using either 10 N KOH or concentrated HCl.

To determine the effect of alternative solvents on the ^{13}C chemical shifts of α-keto acids, phenylpyruvic acid or pyruvic acid (300 mM) was dissolved in 0.5 mL of the appropriate deuterated solvent. [^{1}H]^{13}C NMR spectra were accumulated on a Bruker AM-600 spectrometer using the same parameters as above, with the number of scans ranging from 120 to 320.

Determination of B. subtilis CM Molecular Mass. Three different gel filtration techniques were used to determine the native molecular mass of *B. subtilis* CM. In one case, as part of the CM purification, 144 mg of protein, at a concentration of 7.6 mg/mL, in a buffer A containing ~0.2 M NaCl was loaded onto a 5 × 91 cm Sephacryl S-100 column, and the enzyme was eluted at 1.5 mL/min. The gel filtration standards, which contained ribonuclease A (13.7 kDa), chymotrypsinogen A (25.0 kDa), ovalbumin (43.0 kDa), and bovine serum albumin (67.0 kDa), were run under the same conditions at a concentration of 1.8–2.1 mg of each protein/mL. The second method utilized a FPLC column on an LKB Pharmacia FPLC system. Sephacryl S-100-purified *B. subtilis* CM (1–2 mg) was loaded onto Superdex 70 and eluted under three different conditions: (1) 50 mM KPi and 0.2 M NaCl, pH 7.6; (2) 50 mM KPi and 0.1 M NaCl, pH 7.6; and (3) 50 mM KPi and 0.2 mM prephenate, pH 7.6. In the third method, *B. subtilis* CM (6 mg/mL protein) was loaded onto a Zorbax GF-450 column attached to a Waters 600E HPLC system equipped with a Waters 994 diode array detector; CM was eluted with buffer A with and without the inclusion of 0.2 M NaCl. Bio-Rad gel filtration standards, which contained bovine thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa), and cyanocobalamin (1.35 kDa), were used to calibrate the Superdex and Zorbax columns.

Electrospray mass spectra were recorded on a Sciex API-III triple quadrupole mass spectrometer fitted with a standard pneumatically assisted nebulization probe and an atmospheric pressure ionization source (Sciex, Thornhill, Ontario, Canada). The sample for electrospray mass spectrometry analysis was prepared as follows: 130 μL of *B. subtilis* CM (~8 mg/mL) in buffer A containing 0.2 M NaCl was dialyzed against three changes of 15 mM ammonium bicarbonate buffer (250-, 250-, and 400-mL dialysis volumes). An aliquot of the sample was diluted to a concentration of ca. 50 pmol/μL with methanol/water (50:50 v/v) containing 0.2% formic acid. The sample was introduced into the mass spectrometer by infusion with a syringe pump (Harvard Instruments) at 2 μL/min. Approximately 2 min of data was recorded, and ca. 100 pmol of the sample was consumed.

Table I: Purification of *B. subtilis* CM from 15 g of *E. coli* XL1-BLUE (pBSCM2)

	total protein (mg)	total act. (μmol min^{-1})	sp act. (μmol min^{-1} mg^{-1})	purification factor	act. yield (%)	CM/PD act. ratio
crude extract	690	39 300	57	1	100	5×10^3
CDR	473	41 108	87	1.5	105	1×10^4
DE-52 cellulose	144	29 422	204	3.6	75	2.3×10^5
Sephacryl S-100	89	26 642	324	5.7	68	1×10^6

RESULTS

Overproduction and Purification of B. subtilis CM. Table I describes the purification of *B. subtilis* CM from *E. coli* XL1-Blue (pBSCM2); the crude extract contains 2600 μmol min^{-1} of CM activity/g of cells. The remarkably simple purification, originally devised by Gray et al. (1990b), takes advantage of the fact that most *E. coli* proteins stick to DEAE-cellulose while *B. subtilis* CM does not. From a crude preparation of lysed cells, the DEAE-cellulose column purification yields nearly homogeneous *B. subtilis* CM. Unfortunately, a minor contaminant is CMPD, a bifunctional *E. coli* protein which can convert prephenate to phenylpyruvate. The published purification procedure has been modified first by adding a DEAE batch purification (using CDR) prior to the DE-52 column (Table I), thus extending the column life of the latter. Additional modifications include eliminating one of the ion-exchange columns and adding a gel filtration step. Taking advantage of the fact that *E. coli* CMPD (82 kDa) is twice as big as *B. subtilis* CM, purification on Sephacryl S-100 reduces the relative PD activity to ~10^{-6} that of CM, conditions under which the ^{13}C NMR experiments are feasible (see below). The specific activity of *B. subtilis* CM (324 μmol min^{-1} mg^{-1}) and percent yield (68%) obtained by using this modified procedure are both ~30% higher than those of the previously published procedure. Additional passes through the S-100 column result in CM/PD ratios of up to 4×10^6 with some loss in overall yield.

Determination of B. subtilis CM Molecular Mass. The subunit molecular mass of the cloned *B. subtilis* CM, ~14.3 kDa, on the basis of SDS–PAGE, is in agreement with the known sequence of 124 amino acids. As isolated from *B. subtilis*, CM is reported to be a homodimer (Gray et al., 1990b). In that report, the native protein molecular mass was determined by HPLC in a buffer of low ionic strength which is contraindicated for gel filtration columns. Because of the importance of molecular size on ^{13}C NMR line widths, we have determined the native molecular mass of the cloned *B. subtilis* CM on gel filtration columns of three different

Table II: Determination of Native Molecular Mass of *B. subtilis* CM by Gel Permeation Methods

column	buffer	mol mass (kDa)
Sephacryl S-100	buffer A + 0.2 M NaCl	46
Superdex 70	50 mM KPi + 0.1 M NaCl + 1 mM DTE	48
	buffer A + 0.2 M NaCl	38
	buffer A + 0.2 M NaCl + 0.2 mM prephenate	42
Zorbax GF-450	buffer A	40
	buffer A + 0.2 M NaCl	44

chemistries, Sephacryl S-100 (low pressure), Superdex 70 (FPLC), and Zorbax GF-450 (HPLC), under a variety of conditions as shown in Table II. The molecular masses thus obtained were *all* in the range 38–48 kDa. In all cases, the peak corresponding to *B. subtilis* CM activity occurs immediately after the ovalbumin peak (43 kDa). Although gel permeation is a relatively crude method for molecular mass determination, the CM data agrees better with a homotrimer quaternary structure (43.5 kDa) rather than a dimeric structure (29 kDa). An earlier report of a 40-kDa molecular mass for *B. subtilis* CM as determined by gel permeation (Llewellyn et al., 1980) agrees with our results.

The discrepancy between the molecular mass data presented in Table II and that obtained by Gray et al. (1990b) led us to seek a more precise analysis of the subunit molecular mass and homogeneity of *B. subtilis* CM using electrospray mass spectrometry. This method can be used to detect any posttranslational modification or the presence of any proteolytically cleaved fragments that could associate with the holoenzyme resulting in a higher native molecular mass, yet that might not be revealed by SDS–PAGE. Electrospray mass spectrometry produces a coherent series of multiply charged molecular ions for each protein species present in the sample (Covey et al., 1988; Fenn et al., 1989; Smith et al., 1990). If the mass of the charge-carrying species is known (often H = 1.0079 amu), then the number of charges and the molecular mass can easily be determined. Furthermore, each peak in a coherent series provides an independent measurement of the molecular mass. The subunit molecular mass of *B. subtilis* CM measured by electrospray mass spectrometry is 14 487 ± 1 Da; this is in agreement with the subunit molecular mass of 14 488 Da as calculated from the amino acid sequence (Gray et al., 1990b). Additional peaks of higher mass in each cluster of the mass spectrum of *B. subtilis* CM correspond to replacement of one or more protons with sodium (data not shown). Electrospray mass spectrometry also confirmed the homogeneity of *B. subtilis* CM; there were no components of significantly higher or lower mass and no apparent disulfide linkages.

^{13}C NMR Studies on B. subtilis CM Using [U-^{13}C]-Chorismate, [2,6,9-^{13}C]Chorismate, and [1,3,5,8-^{13}C]Chorismate as Substrate. The ^{13}C NMR spectrum of *B. subtilis* CM, illustrated in Figure 2A, is typical for a protein: aliphatic resonances between 10 and 80 ppm, aromatic resonances at 120–138 ppm, tyrosine and arginine ζ resonances at about 158 ppm, and carbonyl resonances from 167 to 185 ppm. The intrinsic line widths of the protein signals are approximately 15 Hz. The relatively sharp signals at 36.9, 56.6, 127.8, 129.3, and 129.6 ppm (◊) are due to the presence of 3 mM L-phenylalanine which is used to suppress any residual PD activity (Dopheide et al., 1972). In contrast to bifunctional *E. coli* CMs, *B. subtilis* CM is not feedback-inhibited by either phenylalanine or tyrosine (Lorence & Nester, 1967).

Addition of a substoichiometric amount of [U-^{13}C]chorismate (3.5 mN *B. subtilis* CM:1.5 mM chorismate) yields the spectrum of the enzyme-bound [U-^{13}C]prephenate complex (Figure 2B). Under these experimental conditions, on the basis of a K_d of 70 μM (Gray et al., 1990a), virtually all the prephenate is bound to the enzyme. The new resonances (▼) can be identified simply by comparison with the protein spectrum (Figure 2A), even in the crowded aliphatic region. Figure 2C shows the difference spectrum (Figure 2, B – A) clearly indicating the bound prephenate resonances. A small amount of phenylpyruvate was formed due to contaminating PD activity, as indicated by the HCO_3^- resonance at 160.5 ppm. The enzyme-bound resonances are summarized in Table III; most can be assigned to a particular functional group on the basis of chemical shifts. For example, the resonance at 202.8 ppm corresponds to a keto resonance; those between 170 and 180 ppm correspond to carboxylate resonances; 127–132-ppm signals correspond to C_2, C_3, C_5, and C_6 resonances; and the rest correspond to aliphatic resonances. Bound C_7, C_8, and C_9 resonances were assigned by Gray et al. (1990a); therefore, the remaining carboxylate resonance at 177.9 ppm is assigned to C_{10}, and the aliphatic resonance at 49.0 ppm is assigned to C_1. The keto and carboxyl resonances from C_8, C_7, and C_{10} of bound prephenate are much sharper than the methine or methylene resonances because of the absence of dipolar relaxation by directly attached protons. In fact, they are so sharp that the one-bond ^{13}C–^{13}C coupling constants could be obtained (see Table III). Since the intrinsic line widths are ~15 Hz, it is not surprising that we are not able to observe $^2J_{C7,C9}$ in bound prephenate (in free prephenate $^2J_{C7,C9}$ = 12 Hz). The resonance from C_1 appears broader than the other quarternary carbons because it is coupled to five other carbons, C_{10}, C_9, C_6, C_2, and C_4.

The above assignments are further confirmed by studies with [2,6,9-^{13}C]prephenate and [1,3,5,8-^{13}C]prephenate bound to *B. subtilis* CM. The difference spectrum of bound [2,6,9-^{13}C]prephenate is shown in Figure 2D. In addition to confirmation of the C_9 resonance assignment, the nondegeneracy of C_2 and C_6 upon enzyme binding is clearly seen. The resonances at 129.4 and 125.1 ppm are tentatively assigned to C_2 and C_6, respectively, on the basis of the somewhat higher ^{13}C enrichment at C_2 relative to C_6 (36% vs 24%). Because the difference in intensity is relatively small, these assignments must be confirmed using prephenate labeled at either C_2 or C_6. Figure 2E represents the difference spectrum of bound [1,3,5,8-^{13}C]prephenate. This spectrum shows two sharp resonances arising from C_1 and C_8; the signal from C_1 has a narrow line width as expected in the absence of scalar couplings. The nondegeneracy seen above for C_2 and C_6 dictates the nondegeneracy of C_3 and C_5 upon binding the enzyme. On the basis of the relative ^{13}C enrichment (C_3:C_5 = 1:2.3), the resonances at 129.4 and 133.5 ppm are tentatively assigned to C_3 and C_5, respectively. The combination of low ^{13}C enrichment on C_3 (14%) and the presence of a directly attached proton could explain the reduced intensity of the putative C_3 resonance.

Exchange-Rate Studies. In order to determine the exchange rate of free and bound prephenates, the ^{13}C NMR spectra were acquired with excess prephenate as shown in Figure 3. On the basis of a K_d of 70 μM (Gray et al., 1990a), the amount of free prephenate is expected to be in 1.6 times molar excess to bound prephenate. Figure 3A, obtained at 25 °C and 75 MHz, shows the enzyme-bound prephenate in intermediate exchange with free prephenate. Lowering the temperature to 8 °C (Figure 3B) resolves the bound and free prephenate

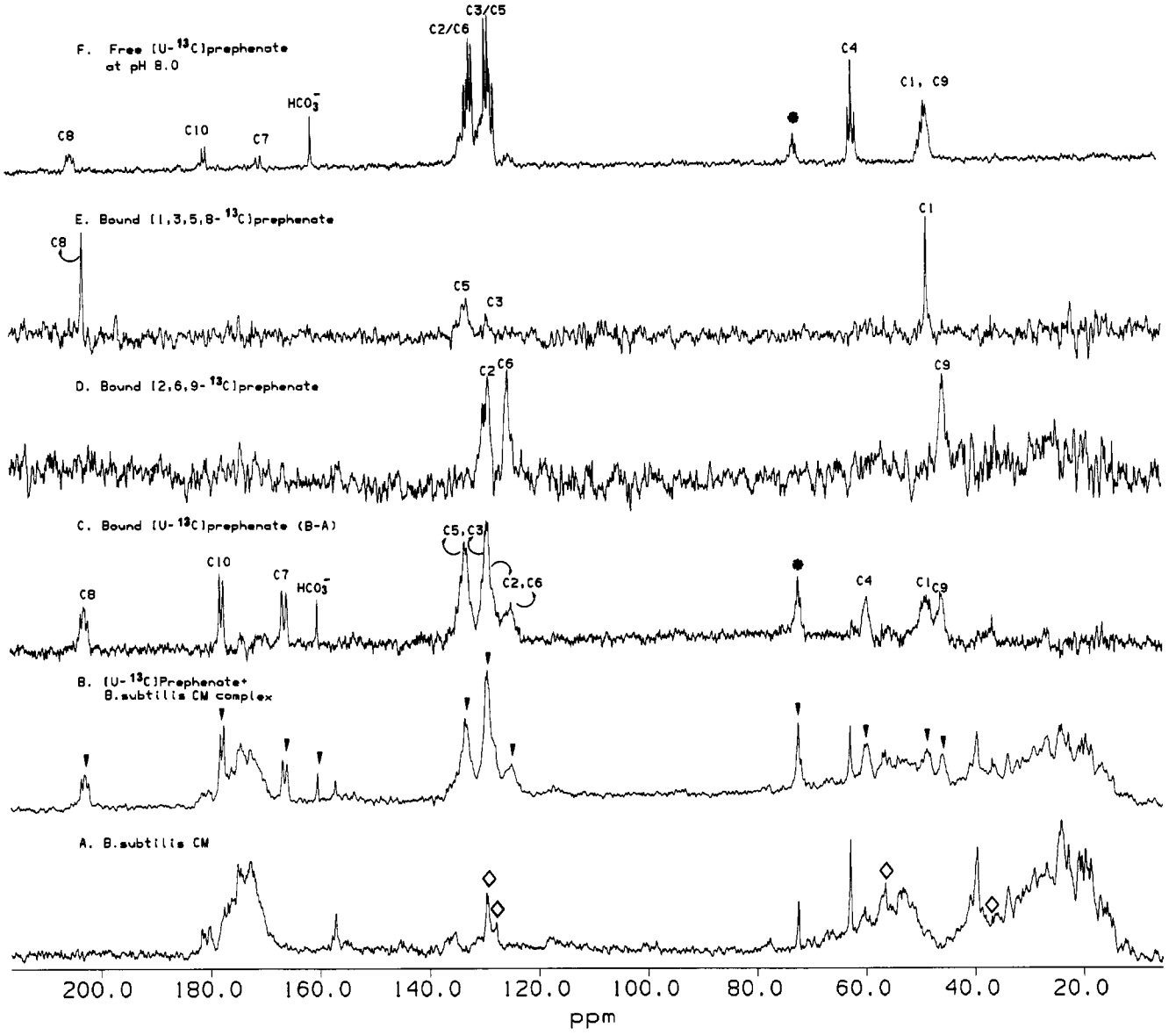

FIGURE 2: [^{1}H]^{13}C NMR spectra: (A) *B. subtilis* CM in a solution containing 3 mM L-phenylalanine (16 000 scans); (B) *B. subtilis* CM–[U-^{13}C]prephenate complex (16 000 scans); (C) difference spectrum of enzyme-bound [U-^{13}C]prephenate (B – A); (D) difference spectrum of enzyme-bound [2,6,9-^{13}C]prephenate (22 000 scans); (E) difference spectrum of enzyme-bound [1,3,5,8-^{13}C]prephenate (18 000 scans); (F) free [U-^{13}C]prephenate (1.5 mM) at pH 8.0 (12 000 scans) (symbols: * from substrate impurity, ◊ phenylalanine, and ▼ new resonances upon addition of [U-^{13}C]chorismate to CM). The NMR sample shown in (A) contained 3.5 mN CM and was not purified on Sephacryl S-100. [U-^{13}C]chorismate was added to the above NMR sample to a final concentration of 1.5 mM (spectrum B). *B. subtilis* CM purified on Sephacryl S-100 was used in subsequent experiments and produced little phenylpyruvate under the above conditions. 0.7 mM [2,6,9-^{13}C]chorismate and 0.75 mM [1,3,5,8-^{13}C]chorismate were used with 1.5 mN *B. subtilis* CM in the experiments shown in (D) and (E), respectively. The [^{1}H]^{13}C NMR spectrum of the CM used in (D) and (E) is not shown. The sample volume was ~2.2 mL in a 10-mm NMR tube for (A)–(E), and the sample volume for (F) was 400 μL in a 5-mm NMR tube. All the spectra were obtained in 50 mM KPi, at pH 8.0 and 25 °C. The spectra were obtained at 75 MHz and processed with 15-Hz line broadening.

resonances of C_4, C_7, and C_{10}, thus indicating slow exchange. Alternative to lowering the temperature, the slow-exchange regime can be achieved by acquiring the spectra at a higher frequency, 150 MHz (Figure 3C), which causes the free and bound resonances to be better resolved. Other prephenate resonances, for example, C_8, are not clearly resolved because multiple coupling patterns cause severe overlap between free and bound resonances. From the data obtained at 150 MHz, the calculated upper limit on the exchange rate between free and bound prephenate at 25 °C is 270 s^{-1}. This is in general agreement with an off-rate of 60 s^{-1} at 30 °C for prephenate bound at the active site of *B. subtilis* CM as obtained by Gray et al. (1990a). For *B. subtilis* CM with a specific activity of 324 μmol min^{-1} mg^{-1} (see Table I), k_{cat} = 78 s^{-1} per subunit at 30 °C.

Modeling the Observed Enzyme-Bound Chemical Shifts. Significant chemical shift changes are observed in all the carbons of CM-bound [U-^{13}C]prephenate relative to free prephenate (Table III). As shown by comparison of spectra C and F in Figure 2, most of the prephenate resonances shift upfield upon binding to the enzyme except for C_1, C_3 and C_5. It is interesting to note that the relative placements of the C_6 and C_5 resonances are switched when prephenate is bound to the enzyme, while the C_2 and C_3 resonances become coincident. We have attempted to rationalize the observed changes in prephenate chemical shifts upon enzyme binding by looking at the pH dependence and solvent effects of various model systems. As illustrated in Table IV, protonation of aliphatic carboxylate anions results in considerable changes in chemical shifts (Breitmaier & Voelter, 1987). Since the shifts of

Table III: ^{13}C NMR Chemical Shifts of Bound and Free [U-^{13}C]prephenate

carbon	free (ppm)	bound (ppm)	change in chemical shift ($\Delta\delta$)	$\Delta J_{C,C}$ (bound − free) (Hz)
C_1	48.6	49.0	0.4	
C_2	131.7	129.4[a]	−2.3	
C_3	127.9	129.4[a]	1.5	
C_4	61.6	59.8	−1.8	$^1J_{C7,C8} = 0 \pm 1$
C_5	127.9	133.5[a]	5.6	
C_6	131.7	125.1[a]	−6.6	
C_7	170.1	166.5	−3.6	$^1J_{C8,C9} = 4.5 \pm 1.5$
C_8	204.3	202.9	−1.5	
C_9	48.1	46.2	−1.9	
C_{10}	180.0	177.9	−2.1	$^1J_{C10,C1} = 5 \pm 1$

[a] Not absolute assignments; see text for details.

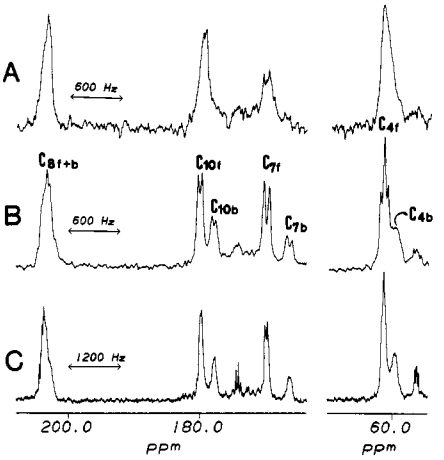

FIGURE 3: Exchange of free (f) and bound (b) [U-^{13}C]prephenate. [^1H]^{13}C spectra of the *B. subtilis* CM–[U-^{13}C]prephenate complex were obtained under the following conditions: (A) 75 MHz, 25 °C (3600 scans); (B) 75 MHz, 8 °C (10 000 scans); (C) 150 MHz, 25 °C (5200 scans). The sample used in NMR exchange studies contained 5.8 mN *B. subtilis* CM and 15 mM [U-^{13}C]prephenate in a 5-mm NMR tube. The spectra were processed with 15-Hz line broadening.

Table IV: Changes in ^{13}C Chemical Shift upon Protonation of Carboxylic Acids and Changing Solvents

	COOH ($\Delta\delta$)	C_α ($\Delta\delta$)	C_β ($\Delta\delta$)	C_γ ($\Delta\delta$)
aliphatic acid, HOOC–C–C–C	−4.7	−3.5	−1.6	−0.6
pyruvate, $HOOCCOCH_3$	−4.9	−5.9	−0.5	na[a]
pyruvic acid[b]				
in $CHCl_3$-*d*	−6.2	−6.8	−3.3	na[a]
in DMSO-d_6	1.2	−0.7	3.4	na[a]
in acetone-d_6	0.1	−1.8	2.1	na[a]
phenylpyruvate, $HOOCCOCH_2$-ϕ	−2.9	−3.5	−0.5	na[a]
enzyme-bound vs free prephenate[c]	−3.6 (C_7)	−1.5 (C_8)	−1.9 (C_9)	0.4 (C_1)
	−2.1 (C_{10})	0.4 (C_1)	−2.3 (C_2)	1.5 (C_3)
			−6.6 (C_6)	5.6 (C_5)
			−1.9 (C_9)	−1.5 (C_8)

[a] Not applicable. [b] Reacts with MeOH-d_4. [c] pH titration of prephenate was not possible because it degrades at pH 6.5 and below.

aliphatic acids may be different from those of keto carboxylic acids, we modeled the changes in chemical shifts of prephenate by pH titration of phenylpyruvate and pyruvic acid (see Table IV). In the case of prephenate itself there are no discernible changes in the ^{13}C spectrum between pH 7 and 13. Below pH 6.5, the carbonyl resonance broadens and the titration is hampered by chemical degradation of prephenate to phenylpyruvate. The solution pK_as of C_7 and C_{10} of prephenate are reported to be 1.4 and 4.2, respectively (Hermes et al., 1984), on the basis of the rates of the acid-catalyzed decarboxylation of prephenate as a function of pH. Solvent effects on ^{13}C NMR chemical shifts of carboxylic acids depend on the extent of hydrogen bonding, but for nonpolar groups solvent effects can differ in magnitude and direction (Breitmaier & Voelter, 1987). Prephenate did not lend itself to the solvent effect studies, because the stable dianion form is insoluble in common organic solvents and decomposes in DMSO. Phenylpyruvic acid exists in the enolic form in DMSO, methanol, and acetone and does not dissolve in chloroform. In the case of pyruvic acid, all the ^{13}C resonances shift upfield in $CHCl_3$-*d* relative to D_2O, whereas the DMSO and acetone the directions of the shifts are not uniform, as shown in Table IV.

DISCUSSION

^{13}C NMR spectra of enzyme-bound ligands can provide valuable structural information, if several stringent requirements are met: availability, stability, and purity of both the ligand and the enzyme. [U-^{13}C]Chorismate, [1,3,5,8-^{13}C]-chorismate, and [2,6,9-^{13}C]chorismate have been prepared by improved methods which provide large quantities of the ^{13}C-labeled chorismate in a sufficiently pure form (Rajagopalan et al., 1992, Rajagopalan & Jaffe, 1993). Prephenate, the predominant enzyme-bound ligand, is sufficiently stable under experimental conditions (pH 8). *B. subtilis* CM is abundantly available through overproduction in *E. coli* using the method of Gray et al. (1990b). *B. subtilis* CM retains more than 90% of its activity after at least 2 days at room temperature. Enzyme purity is another concern. The ^{13}C NMR experiments, which use stoichiometric amounts of enzyme and substrate for long periods of time, are hampered if any contaminating enzymes degrade the protein or metabolize the ligand. Thus, proteases must be scrupulously avoided along with contaminating enzymes which can act on the ligand of interest. Optimally, the ratio of the activity of interest to any contaminating activity should be ca. 10^6; this allows the execution of multiple NMR experiments, on each protein sample, over a period of several days. Our modified purification of *B. subtilis* CM is designed to achieve this goal (see Table I). Consequently, the ^{13}C NMR spectra of enzyme-bound [U-^{13}C]prephenate illustrated in Figure 2 are of strikingly better quality than the previously published spectra of *B. subtilis* CM-bound [7,8,9-^{13}C]prephenate (Gray et al., 1990a). In addition to the reduced PD levels, our modifications include the following: (1) most of the studies described here used a 7T instrument (75 MHz for ^{13}C) because sharper lines can be obtained at lower frequency; (2) NMR spectra were obtained at 25 °C to further decrease the rotational correlation time; and (3) phenylalanine was used to suppress the minute residual PD activity.

As illustrated in Tables III and IV, simple model studies do not accurately reflect the changes in chemical shifts of prephenate bound to CM. Looking first at the pyruvoyl side chain (C_7–C_8–C_9) relative to pyruvate or phenylpyruvate, the directions, but not the magnitudes, of the chemical shift changes are consistent with protonation of the C_7 carboxylic acid. This may reflect partial protonation of the enzyme-bound species. It is clear that the pyruvoyl side chain exists mainly in the keto, not the enolic, form on the basis of the chemical shifts of the bound C_7 resonance. In the case of the C_{10} carboxylic acid, again the chemical shift change is consistent with at least partial protonation, but this effect is

not transmitted to C_1. However, we note that there is no readily available model compound for studying the effects of pH on the chemical shifts at the cyclohexadienyl portion of prephenate. One of our most exciting results is that we can observe and quantify the one-bond couplings of enzyme-bound prephenate; $^1J_{C10,C1}$ increases about 5 Hz upon enzyme binding. Protonation of a carboxylic acid generally increases $^1J_{C,C}$ by about 7 Hz (Breitmaier & Voelter, 1987; Rajagopalan et al., 1992). Thus, both the chemical shift and the coupling constant of C_{10} are consistent with partial protonation. In solution, the decarboxylation of prephenate is acid-catalyzed, driven by protonation of the C_4 hydroxyl group and assisted by ionization of the C_{10} carboxylic acid (Hermes et al., 1984). For enzyme-bound prephenate, the upfield shift at C_4 argues against protonation of the C_4 hydroxyl group and C_{10} appears at least partially protonated. Both of these observations support an active site model which protects prephenate from decarboxylation.

It is clear from the nondegeneracy of C_2 and C_6 and of C_3 and C_5 that the asymmetry of the active site contributes to the observed changes in chemical shifts of enzyme-bound prephenate. It is also interesting to note that the changes in chemical shifts of C_2 and C_3 or C_6 and C_5 are almost equal in magnitude and shift in opposite directions. This might be an indication of polarization of the olefinic bonds, for example, inducing a positive charge on C_3 or C_5 and negative charge on C_2 or C_6 and spreading the effect to C_1 and C_4. The changes in chemical shifts of enzyme-bound prephenate may also arise from the presence of hydrophobic, aromatic, or hydrogen-bonding groups interacting with prephenate. Although it is conceivable to mimic the effect of a symmetric hydrophobic active site by the use of organic solvents, in this case only the model compound pyruvic acid lent itself to solvent studies (see Results). It must be emphasized that the trend of solvent effect on ^{13}C NMR chemical shifts is not always predictable. The trend in changes of ^{13}C NMR chemical shifts of pyruvic acid in $CHCl_3$-*d* is similar to that of the pyruvoyl side chain of enzyme-bound prephenate, which is different from that of pyruvic acid in hydrogen-bond-acceptor solvents (DMSO and acetone). The changes in the chemical shifts of carboxylic acids might be due to close association of the C_7 or the C_{10} carboxylate anion with an electrophilic (protonated) amino acid side chain (e.g., lysine). Although *B. subtilis* CM is not rich in aromatic amino acids (4/subunit), specific anisotropic interactions between side-chain aromatic groups and the olefinic region of prephenate might induce the observed changes in chemical shifts.

Additional contributions to the changes in chemical shifts and coupling constants of enzyme-bound prephenate may derive from a distorted conformation for the enzyme-bound species. The olefinic region of enzyme-bound prephenate shows significant perturbation compared to free prephenate (Table III) with an upfield shift of 6.6 ppm for the C_6 resonance and a downfield shift of 5.4 ppm for the adjacent C_5 resonance. An additional contribution to the observed data may be the relative orientation of the pyruvoyl moiety with respect to the olefinic bonds which can result in the shielding or deshielding of the olefinic resonances.

The ^{13}C NMR studies indicate that the predominant enzyme-bound ligand is prephenate. Gray et al. (1990a) observed that when a transition-state analogue, a competitive inhibitor of CM, was added to the *B. subtilis* CM–prephenate complex, enzyme-bound prephenate was displaced into solution. Our ^{13}C NMR studies were done under similar conditions, and therefore, we conclude that prephenate is bound at the active site of the enzyme. There is no ^{13}C NMR evidence supporting significant population of the enol(ate) tautomer of prephenate, nor is there any evidence for a dissociative intermediate (enolpyruvate moiety from the side chain and a covalently enzyme-bound cyclohexadienyl portion of prephenate) as proposed by Guilford et al. (1987). To further probe for the existence of these species, we monitored the exchange of the C_9 protons of prephenate in D_2O. In the absence of CM, at pH 8, the C_9 protons do not exchange, indicating that the enol(ate) tautomer of prephenate is not populated in solution. In the presence of CM, at a CM:prephenate ratio of 1:50 (starting from chorismate), the C_9 protons of prephenate again show no inclination toward exchange over 24 h at 25 °C. Although it remains possible that an enzyme-bound enol(ate) may be sufficiently shielded from solvent to prevent proton exchange, our data provide no evidence for the enol(ate) tautomer of prephenate for the enzyme-bound species. The data also provide no support for a dissociative mechanism which involves a bound enolpyruvate intermediate.

An intriguing aspect of CM is the lack of sequence homology between enzymes of different species. The documented CM sequences include two different bifunctional CMs of *E. coli* (Hudson & Davidson, 1984), monofunctional yeast CM (Schmidheini et al., 1989), and monofunctional *B. subtilis* CM (Gray et al., 1990b). The N-terminal half of the bifunctional *E. coli* enzymes is believed to be responsible for the CM reaction (Hudson et al., 1984; Maruya et al., 1987). In order to determine the uniqueness of this sequence diversity, we compared CM with 20 other homomeric enzymes drawn from heme biosynthesis, the citric acid cycle, amino acid metabolism and synthesis, lipid biosynthesis, purine biosynthesis, carbohydrate metabolism, and protein biosynthesis (GCG Wisconsin Sequence Package; Devereaux et al., 1984). Homology among CMs from a variety of sources was minimal and did not differ significantly from the extent of homology among the 20 random sequences. If the role of CM is simply to bind chorismate in an appropriate conformation for an asynchronous concerted reaction, then perhaps sequence homology is not necessary because there are a variety of active site amino acid constellations which can achieve this goal. We plan to characterize the ^{13}C NMR spectra of prephenate bound to CMs from different sources to address this apparent paradox. Should we observe invariant chemical shifts for prephenate bound to CMs of different sequences, this would argue for similar active site structures and mechanisms despite the lack of sequence homology.

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REFERENCES

Addadi, L., Jaffe, E. K., & Knowles, J. R. (1983) *Biochemistry 22*, 4494–4501.

Allerhand, A. (1979) *Methods Enzymol. 61*, 458–549.

Andrews, P. R., Cain, E. N., Rizzardo, E., & Smith, G. D. (1977) *Biochemistry 16*, 4848–4852.

Andrews, P. R., & Haddon, R. C. (1979) *Aust. J. Chem. 32*, 1921–1929.
Andrews, P. R., Smith, G. D., & Young, I. G. (1973) *Biochemistry 12*, 3492–3498.
Barlow, P. N., Appleyard, R. J., Wilson, B. J. O., & Evans, J. N. S. (1989) *Biochemistry 28*, 7985–7991.
Bartlett, P. A., Nakagawa, Y., Johnson, C. R., Reich, S. H., & Luis, A. (1988) *J. Org. Chem. 53*, 3195–3210.
Breitmaier, E., & Voelter, N. (1987) in *Topics in Carbon-13 NMR Spectroscopy*, VHS Publishers, New York.
Clarke, T., Stewart, J. D., & Ganem, B. (1987) *Tetrahedron Lett. 28*, 6253–6256.
Clarke, T., Stewart, J. D., & Ganem, B. (1990) *Tetrahedron 46*, 731–748.
Copley, S. D., & Knowles, J. R. (1985) *J. Am. Chem. Soc. 107*, 5306–5308.
Copley, S. D., & Knowles, J. R. (1987) *J. Am. Chem. Soc. 109*, 5008–5013.
Covey, T. R., Bonner, R. F., Shushan, B. I., & Henion, J. (1988) *Rapid Commun. Mass Spectrom. 2*, 249–255.
Delany, J. J., III, Padykula, R. E., & Berchtold, G. A. (1992) *J. Am. Chem. Soc. 114*, 1394–1397.
Devereaux, J., Haeberli, P., & Smithies, O. (1984) *Nucleic Acids Res. 12*, 387–395.
Dopheide, T. A. A., Crewther, P., & Davidson, B. E. (1972) *J. Biol. Chem. 247*, 4447–4452.
Edwards, J. M., & Jackman, L. M. (1965) *Aust. J. Chem. 18*, 1227–1239.
Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., & Whitehouse, C. M. (1989) *Science 246*, 64–71.
Gajewski, J. J., Jurayj, J., Kimbrough, D. R., Gande, M. E., Ganem, B., & Carpenter, B. K. (1987) *J. Am. Chem. Soc. 109*, 1170–1186.
Ganem, B. (1978) *Tetrahedron 34*, 3353–3383.
Gething, M.-J. H., Davidson, B. E., & Dopheide, T. A. A. (1976) *Eur. J. Biochem. 71*, 317–325.
Gibson, M. I., & Gibson, F. (1964) *Biochem. J. 90*, 248–261.
Gray, J. V., Eren, D., & Knowles, J. R. (1990a) *Biochemistry 29*: 8872–8878.
Gray, J. V., Grolinelli-Pimpaneau, B., & Knowles, J. R. (1990b) *Biochemistry 29*, 376–383.
Guilford, W. J., Copley, S. D., & Knowles, J. R. (1987) *J. Am. Chem. Soc. 109*, 5013–5019.
Hermes, J. D., Tipton, P. A., Fisher, M. A., O'Leary, M. H., Morrison, J. F., & Cleland, W. W. (1984) *Biochemistry 23*, 6263–6275.
Hudson, G. S., & Davidson, B. E. (1984) *J. Mol. Biol. 180*, 1023–1057.
Hudson, G. S., Wong, V., & Davidson, B. E. (1984) *Biochemistry 23*, 6240–6249.
Jaffe, E. K., & Markham, G. D. (1987) *Biochemistry 26*, 4258–4264.
Jaffe, E. K., & Markham, G. D. (1988) *Biochemistry 27*, 4475–4481.
Llewellyn, D. J., Daday, A., & Smith, G. D. (1980) *J. Biol. Chem. 255*, 2077–2084.
Lorence, J. H., & Nester, E. W. (1967) *Biochemistry 6*, 1541–1553.
Malthouse, J. P. G., & Finucane, M. D. (1991) *Biochem. J. 280*, 649–657.
Maruya, A., O'Connor, M. J., & Backman, K. (1987) *J. Bacteriol. 169*, 4852–4853.
Pawlak, J. L., & Berchtold, G. A. (1988) *J. Org. Chem. 53*, 4063–4069.
Pawlak, J. L., Padykula, R. E., Kronis, J. D., Aleksejczyk, R. A., & Berchtold, G. A. (1989) *J. Am. Chem. Soc. 111*, 3374–3381.
Poulsen, C., & Verpoorte, R. (1991) *Phytochemistry 30*, 377–386.
Rajagopalan, J. S., & Jaffe, E. K. (1993) *Symposium-in-Print, The Shikimic Acid Pathway* (Botting, N. P., Ed.) *BioMed. Chem. Lett.* (in press).
Rajagopalan, J. S., Chen, L.-C., & Jaffe, E. K. (1992) *Bioorg. Chem. 20*, 115–123.
Schmidheini, T., Speaisen, P., Paravicini, G., Hütter, R., & Brans, G. (1989) *J. Bacteriol. 171*, 1245–1253.
Schmidheini, T., Mösch, H.-U., Evans, J. N. S., & Braus, G. (1990) *Biochemistry 29*, 3660–3668.
Smith, R. D., Loo, J. A., Edmonds, C. G., Barinaga, C. J., & Udseth, H. R. (1990) *Anal. Chem. 62*, 882–899.
Turnbull, J., Cleland, W. W., & Morrison, J. F. (1991) *Biochemistry 30*, 7777–7782.