Intersubunit Communication in Tryptophan Synthase by Carbon-13 and Fluorine-19 REDOR NMR†

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ABSTRACT: The β subunits of the 143-kDa $\alpha_2\beta_2$ tetrameric enzyme tryptophan synthase have been labeled by L-[ring-4-^{19}F]phenylalanine and L-[phenol-4-^{13}C]tyrosine in an effort to monitor the positions of these residues on ligand binding. Of the 13 phenylalanine and 11 tyrosine residues in the β subunit, only three pairs have labels with ^{13}C−^{19}F separations of less than 6 Å. The β-subunit residues Tyr279 and Phe280 (each members of one of the three Tyr−Phe proximate pairs) have been suggested as possible conformational gates on ligand binding. The 188-MHz ^{19}F NMR spectrum of the microcrystalline, double-labeled enzyme complex has five resolved lines under 5-kHz magic-angle spinning and 80-kHz proton dipolar decoupling. The distribution of β-subunit ^{19}F isotropic shifts is altered by addition of L-[3-^{13}C]-serine to the mother liquor in contact with the microcrystals, consistent with a conformational rearrangement. The ^{13}C label from serine is detected at 28 ppm as a methyl tautomer of bound aminoacrylate. The change in aromatic ^{19}F chemical shifts on binding of serine indicates an alteration in local electric field gradients within the β subunit. However, rotational-echo double-resonance ^{13}C NMR (with ^{19}F dephasing) shows that the average ^{13}C−^{19}F distance for the three phenylalanine−tyrosine proximate pairs in the β subunit is changed by less than 1 Å.

Substrate channeling is a process by which two sequential enzymes interact to transfer a metabolite (or intermediate) from one active site of an enzyme to the next without allowing free diffusion of the metabolite (Welch, 1977). Channeling is thought to play an important role in the metabolic regulation and cellular modulation of enzymatic activities (Srere, 1987; Ovadi, 1988). Tryptophan synthase (Trp-S),[1] a 143-kDa $\alpha_2\beta_2$ tetrameric enzyme complex is a well-documented example of an enzyme which displays channeling behavior between α and β subunits (Demoss, 1962; Creighton, 1970; Matchett, 1974; Miles, 1991). The α subunit catalyzes the cleavage of indole-3-glycerol phosphate (IGP) to indole and glyceraldehyde-3-phosphate (G3P). This reaction is inhibited by indole propanol phosphate (IPP), a competitive inhibitor of IGP (Kirschner et al., 1975). The β subunit catalyzes the condensation of indole with serine to form tryptophan in a reaction mediated by pyridoxal phosphate (PLP). The crystal structure of Trp-S from *Salmonella typhimurium* reveals a 25-Å long hydrophobic tunnel which is thought to serve as a passageway for the transfer of indole from the α to β catalytic sites (Hyde et al., 1988). Trp-S has nearly full catalytic activity in the crystalline state suggesting that channeling between subunits is also present in the crystal (Ahmed et al., 1987; Mozzarelli et al., 1989).

Mechanistic studies with Trp-S have shown that serine, the β-subunit ligand, activates the α subunit for the aldolytic cleavage of IGP to indole (Dunn et al., 1990; Anderson et al., 1991; Lane & Kirschner, 1991). This activation process is mediated by pyridoxal phosphate (PLP); the chemical species involved are summarized in Scheme 1. In the absence of serine, PLP is covalently bound to β-Lys87 (**I**). When serine binds, the amino group of serine replaces the ϵ-amino group of the lysine forming an external aldimine (**II**). This is followed by a deprotonation step to form a quininoid (**III**), which subsequently is protonated and then loses water to form an aminoacrylate (**IV**) that is in equilibrium with a methyl tautomer either **V** or **VI** (Karube & Matsushima, 1976; Kayastha et al., 1991; Schnackerz et al., 1995). Previous transient kinetic studies have established that the steps leading up to the formation of **IV** and **V** or **VI** occur rapidly (Anderson et al., 1991). In the absence of indole, the aminoacrylate species slowly hydrolyzes, most likely through an iminopyruvate, to pyruvate and ammonia. The aminoacrylate in the β subunit is key to the activation of the α subunit (Dunn et al., 1990; Anderson et al., 1991; Lane & Kirschner, 1991). Although this activation process is most likely mediated by a protein conformational change, no structural information from X-ray crystallography has been obtained to date because of the disorder induced within the Trp-S crystals by the addition of serine.

The goals of the current study were first, to demonstrate the applicability of solid-state NMR methods for characterizing internal conformational reorganizations of large proteins and, second, to gain insight into the Trp-S change induced by the binding of serine at the β subunit. It has been suggested that two residues in particular, β-F280 and β-Y279, which are part of the hydrophobic tunnel, might move relative to one another and therefore serve as a gate to

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[1] Abbreviations: Trp-S, tryptophan synthase; IGP, indole-3-glycerol phosphate; G3P, glyceraldehyde 3-phosphate; IPP, indole propanol phosphate; PLP, pyridoxal phosphate; REDOR, rotational-echo double resonance.

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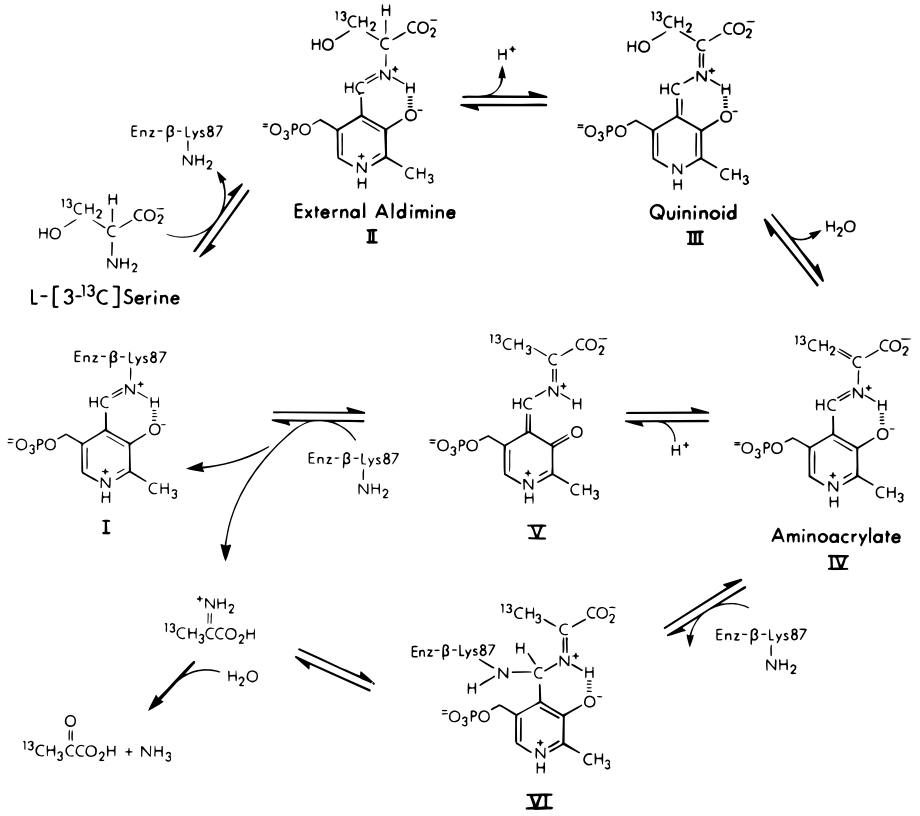
Scheme 1



modulate the passage of indole on binding of serine (Stroud, 1994). These two residues are members of two of the three F−Y pairs in the β subunit that are within 6 Å of each other (as measured from the ring-4 positions). These pairs are F280−Y186, F306−Y279, and F241−Y324. In this paper, we report the detection of a Trp-S bound aminoacrylate intermediate derived from L-[3-^{13}C]serine and changes in the isotropic ^{19}F chemical shifts of α_2-[ring-4-^{19}F]F-[phenol-4-^{13}C]Y-β_2 Trp-S induced by the addition of serine in the absence of IGP. However, rotational-echo double-resonance ^{13}C NMR (with ^{19}F dephasing) shows that the average ^{13}C−^{19}F distance for the three phenylalanine−tyrosine proximate pairs in the β subunit is changed by less than 1 Å.

MATERIALS AND METHODS

Cell Culture. Separate clones for the Trp-S α and β subunits from *S. typhimurium* containing two plasmids (pStrpA and pStrpB) that carry the trp A and trp B genes, respectively, were obtained from Dr. Edith Miles at NIH (Yang et al., 1992). These plasmids contained an ampicillin-resistant gene as a marker. When each of the plasmids was expressed in *E. coli* CB149 overproduction of each individual subunit was attained. To prepare either subunit protein containing isotopically labeled amino acids, the following Vogel−Bonner minimal media recipe was used (per liter): K_2HPO_4, 10 g; $NaNH_4PO_4(4H_2O)$, 3.5 g; citric acid, 2 g; $MgSO_4(7H_2O)$, 0.2 g; glucose, 5 g; L-tryptophan, 5 mg; ampicillin, 30 mg; and 100 mg/L each of the common 20 amino acids. To prepare the β subunit isotopically labeled with L-[phenol-4-^{13}C]tyrosine ([^{13}C]Y) and L-[ring-4-^{19}F]-phenylalanine ([^{19}F]F), 100 mg/L of the 18 unlabeled amino acids as well as 100 mg/L of [^{13}C]Y (^{13}C, 99%, Cambridge Isotope Laboratories) and 400 mg/L of [^{19}F]F (Sigma) were included. The higher amount of [^{19}F]F was required for good isotopic incorporation. When unlabeled subunit (either α or β) was prepared, either the above recipe with 0.5 g of acid-hydrolyzed casein substituted for the amino acids or standard LB-broth containing ampicillin was used.

Trp-S Purification. The method of purification for the α and β subunits was primarily that of Yang et al. (1992). Briefly, after 16−24 h of growth at 37 °C with vigorous aeration, the cells were harvested and broken using a French Press at 20 000 psi. The cellular debris was removed by centrifugation, and the supernatant was fractionated with ammonium sulfate and dialyzed into 50 mM Bicine, 1 mM EDTA, and 1 mM dithiothreitol (DTT), and 0.1 mM pyridoxal phosphate (PLP) at pH 7.8 (buffer A). The protein (either α or β subunit) was chromatographed on an anion exchange column (Q-Sepharose; Pharmacia) using a linear gradient of 0−100% buffer A with 1 M NaCl. After chromatography, the individual purified subunits were concentrated and dialyzed into 50 mM Tris(HCl), 5 mM EDTA, 5 mM β-mercaptoethanol, and 0.1 mM PLP at pH 7.0 (buffer T). The purified subunits were combined in a molar ratio of 2:1 (α to β) to form the $\alpha_2\beta_2$ tetrameric complex. After the solution was stored overnight at 4 °C, the complex was crystallized by adding 30% (w/v) poly-(ethylene glycol) 8000 and spermine in buffer T to give a final concentration of 6% poly(ethylene glycol) 8000 and 5 mM spermine. The microcrystals formed after approximately 48 h were collected by centrifugation at 6000*g*. The tetrameric complex in crystalline form retained catalytic activity (McDowell et al., 1995). The microcrystals were then exchanged into mother liquor of lower ionic strength for NMR experiments [5 mM Tris, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.1 mM PLP, 5 mM spermine, 30% poly-(ethylene glycol) 8000 either with or without 100 mM L-[3-^{13}C]serine (Merck Stable Isotopes), and either with or without

3 or 6 mM IPP]. The resulting ~80 mg sample was packed into a 7.5-mm high-performance zirconia rotor fitted with plastic (Kel-F) endcaps and spacers. The sample was cooled (but not frozen) to a temperature of approximately 5 °C during NMR experiments to compensate for inductive heating.

Computer Modeling. Distances between isotopically labeled nuclei were determined using Sybyl (Tripos Associates, St. Louis, MO) and the protein coordinates (1WSY) from the Brookhaven Protein Data Bank.

Nuclear Magnetic Resonance. Solution-state NMR spectra were obtained using a Varian Unity 300-MHz spectrometer. Solid-state NMR experiments were performed using a home-built spectrometer operating with a 4.7-T wide-bore Oxford magnet (Oxford, England). The four-radiofrequency, quadruple-tuned NMR probe makes use of coaxial transmission lines connecting tuning components in a remote box to a single, seven-turn, 9-mm solenoidal coil in the magnet. The probe uses standing waves to generate impedance null points for joining the tuning components of different frequencies together with no interferences (Holl et al., 1990). The spectrometer has a Chemagnetics (Fort Collins, CO) spinning system, ENI transmitters (Rochester, NY) for frequencies below 100 MHz, and Kalmus transmitters (Woodinville, WA) for the ^{1}H and ^{19}F frequencies. The pulse generator and data acquisition system are from Tecmag (Houston, TX). Specially designed circuits control the radiofrequency pulse amplitude and magic-angle-spinning speed.

REDOR utilizes magic-angle sample spinning to produce high-resolution spectra and measures the heteronuclear dipolar coupling (D_{IS}) between isolated pairs of labeled nuclei, I and S (Gullion & Schaefer, 1989a,b). The basic experiment consists of preparation of transverse S-spin magnetization by cross-polarization of the S spins from the abundant proton reservoir, followed by a period of I−S dipolar evolution that reintroduces the weak heteronuclear dipolar coupling removed by spinning. The S-spin signal is detected. The dipolar evolution period contains two sets of rotor-synchronized, interleaved pulse trains; the first set consists of I-spin π pulses in the middle of each rotor cycle, and the second set consists of S-spin π pulses at the end of each rotor cycle. (Placing I and S π pulses at half-rotor period intervals maximizes the dephasing during the dipolar evolution time.) REDOR requires two spectra to be collected, one with the pulses on the I channel to produce the dephased signal, S, and one without dephasing pulses to produce the full-echo signal, S_0. In a powder, the difference between the two spectra ($\Delta S = S_0 - S$) divided by S_0 can be directly related to the dipolar coupling, D_{IS}, between I and S spins (Pan et al., 1990). Given D_{IS}, the internuclear distance, r_{IS}, can be easily calculated from $r_{IS} = \{(\gamma_I\gamma_S h)/(2\pi D_{IS})\}^{1/3}$ where γ_I and γ_S are the gyromagnetic ratios of the I and S spins, respectively, and h is Planck's constant. Because GHz-regime, ultra-high-frequency motions partially average the dipolar interaction, measured couplings are slightly less than the rigid-lattice values. In crystalline solids this reduction is small (Pan et al., 1990) and is routinely included in distance determinations (McDowell et al., 1993). The ability of REDOR to determine distances in proteins and peptides has been well documented (Marshall et al., 1990; Holl et al., 1992; Christensen & Schaefer, 1993; McDowell et al., 1993; Beusen et al., 1994; Hing et al., 1994; Mueller et al., 1995).

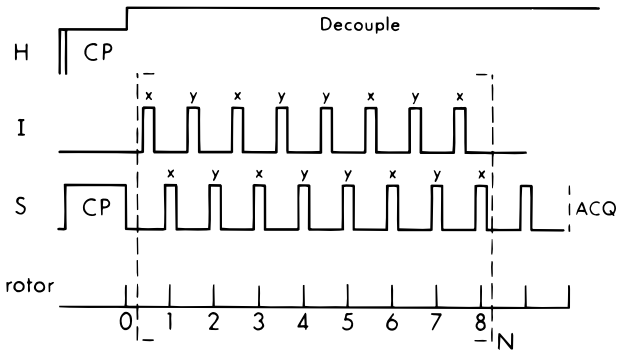


FIGURE 1: REDOR pulse sequence.

Table 1: Distances and REDOR Parameters for α_2-[^{19}F]F-[^{13}C]Y-β_2 Trp-S Based on the X-ray Crystal Structure of Non-Fluorinated Tryptophan Synthase

amino acid pair	^{19}F−^{13}C distance (Å)	dipolar coupling (Hz)	predicted $\Delta S/S_0$ for $N_c = 8$[a]	predicted $\Delta S/S_0$ for $N_c = 24$[a]	predicted $\Delta S/S_0$ for $N_c = 80$[a]
F280−Y186	3.41	697	0.84	0.93	1.00
F306−Y279	4.54	295	0.21	0.99	0.91
F241−Y324	5.02	218	0.12	0.76	0.96
F280[b]−Y279[b]	7.87	57	0	0.07	0.63

[a] Values assume isolated spin pairs and 100% isotopic incorporation of the dephasing nucleus. See McDowell et al. (1993) for the full dependence of $\Delta S/S_0$ on N_c including oscillatory behavior after reaching maximum dephasing. [b] These residues appear in other pairs.

Figure 1 shows the ^{13}C-observe, ^{19}F-dephase REDOR pulse sequence. The XY8 phase cycling (Gullion et al., 1990) minimizes effects due to off-resonance imperfect pulses. Two extra rotor cycles (and a Hahn refocusing pulse) are added to the end of the sequence so that data acquisition is not coincident with a pulse. To obtain ^{13}C REDOR NMR spectra, matched spin-lock cross-polarization transfers were performed in 2 ms at 38 kHz, ^{13}C and ^{19}F π pulses were produced by 38 and 50-kHz radiofrequency fields, respectively, and proton decoupling was at 80 kHz. Distance measurements using ^{13}C REDOR with ^{19}F dephasing were calibrated using ^{19}FCH$_2$CO-[1-^{13}C]MeA^4-[^{15}N]Val^5-emerimicin 1−9 (Holl et al., 1992). To obtain ^{19}F REDOR NMR spectra, cross-polarization transfers were performed in 0.4 ms at 50 kHz, with the other radiofrequency amplitudes the same as for the ^{13}C-observe experiments. Acquisitions alternated between dephased (S) and full-echo (S_0) spectra with a repetition period of 2 s. Spectra obtained with magic-angle spinning have spinning side bands spaced at integral multiples of the spinning speed from the center band. If the chemical shift anisotropy is large, the percentage of spectral intensity contained in the side bands is significant and must be included in the REDOR calculations.

RESULTS

Interatomic Distances in Trp-S. Using the X-ray crystal structure previously determined for *S. typhimurium* as a starting point, distances were calculated between each tyrosine and fluorophenylalanine pair in the β subunit. According to the amino acid sequence for the β subunit, 13 phenylalanine residues and 11 tyrosine residues are labeled. Only four F−Y pairs are closer than 8 Å (Table 1). The α-carbon backbone of an $\alpha\beta$ dimer and the side chains of the three closest F−Y pairs in the β subunit are shown in Figure 2.

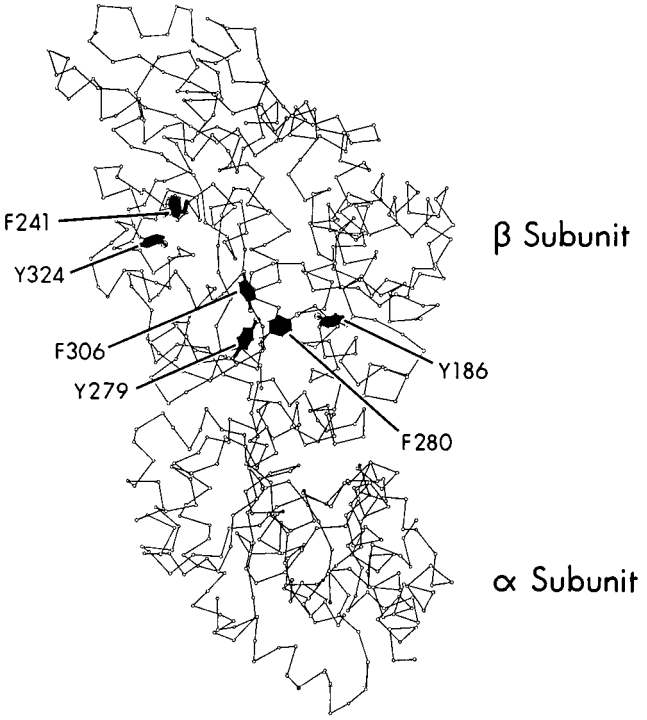


FIGURE 2: Crystal structure of the $\alpha\beta$ dimer of tryptophan synthase showing the positions of the phenylalanine and tyrosine residues that form proximate pairs near the β-subunit active site.

Protein and Protein Complex Characterization by ^{19}F and ^{13}C NMR. The Trp-S $\alpha_2\beta_2$ tetramer with β subunits labeled specifically by [^{19}F]F and [^{13}C]Y has a steady-state turnover rate similar to that observed for unlabeled wild type Trp-S (data not shown). The [^{19}F]F NMR intensity of Trp-S α_2-[^{19}F]F-[^{13}C]Y-β_2 was compared to the ^{19}F signal intensity of poly(*p*-fluorostyrene), a poly-*p*-fluorostyrene(10%)−polystyrene(90%) copolymer (Tong et al., 1995), crystalline L-[4-^{19}F]phenylalanine, and crystalline D,L-[6-^{19}F]tryptophan. Each of the reference materials contained a known amount of fluorine. Calibration and analytical experiments were performed consecutively. These comparisons established a [^{19}F]F label fraction in Trp-S of 0.37 ± 0.07.

The relative intensities of the carbonyl-carbon peak (175 ppm) and the [^{13}C]Y peak (156 ppm) in a two rotor-cycle spin-echo spectrum (not shown) were used to estimate the fraction of [^{13}C]Y incorporation (Jacob et al., 1985; McDowell et al., 1993) in α_2-[^{19}F]F-[^{13}C]Y-β_2. Contributions to the intensity in the 156 ppm region arise from ^{13}C label in the 11 tyrosines in each β subunit and from the 1.1% natural-abundance ^{13}C in the six tyrosines in each α subunit (155 and 157 ppm), as well as the guanidino carbons of the 33 arginines in α and β subunits (155 ppm). Contributions to the intensity in the 175 ppm region arise from the natural-abundance ^{13}C in the 738 backbone and side-chain carbonyl carbons in each $\alpha\beta$ dimer. The experimental ratio of [^{13}C]Y to carbonyl-carbon integrated intensity was 1.18, which led to an estimated [^{13}C]Y label fraction in Trp-S of 0.86 ± 0.09. The ability to use integrated signal intensities calibrated either externally or internally to determine isotopic enrichment nondestructively on the identical sample used for structural experiments is an important attribute of cross-polarization, magic-angle spinning NMR (Schaefer & Stejskal, 1976).

When Trp-S α_2-[^{19}F]F-[^{13}C]Y-β_2 was mixed with a solution of L-[3-^{13}C]serine, a ^{13}C resonance due to the serine label

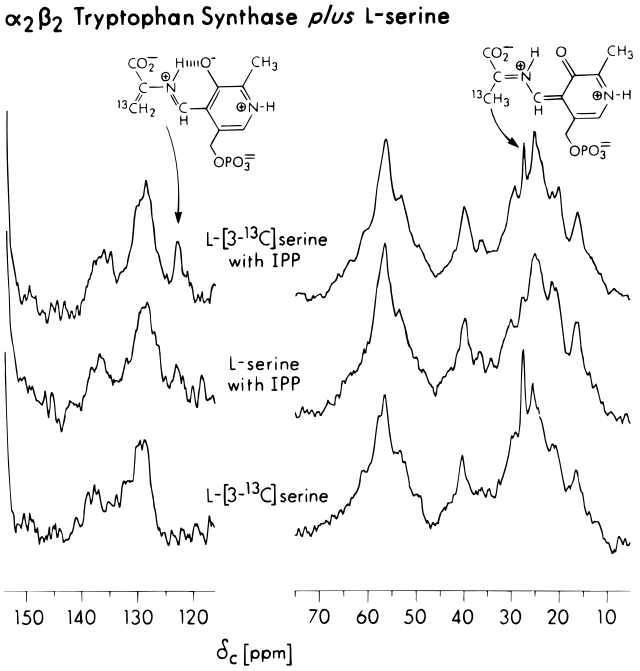


FIGURE 3: 50-MHz ^{13}C NMR spectra of α_2-[ring-4-^{19}F]phenylalanine-[phenol-4-^{13}C]tyrosine-β_2 tryptophan synthase with 5-kHz magic-angle spinning and 80-kHz proton dipolar decoupling. The mother liquor bathing the microcrystals contained (top) 100 mM L-[3-^{13}C]serine and 3 mM indole propanol phosphate (IPP), (middle) 100 mL L-serine (unlabeled) and 6 mM IPP, or (bottom) 100 mM L-[3-^{13}C]serine. Cross-polarization magic-angle spinning Hahn-echo spectra are shown on the right, and 24 rotor-cycle REDOR full-echo spectra are on the left. Because of differential relaxation, the REDOR spectra are better resolved than the Hahn-echo spectra in the vinyl-carbon region. The 28 ppm peak in the top and bottom spectra is assigned to ^{13}C-labeled methyl ketoamine tautomer (**V** or **VI**) from metabolism of L-[3-^{13}C]serine (Scheme 1). The peak at 123 ppm in the top spectrum may indicate a bound [^{13}C]-aminoacrylate and is assigned to **IV** (Scheme 1). The 2-ms proton-to-carbon cross-polarization transfer used for both Hahn-echo and REDOR full-echo spectra ensures that unbound L-[3-^{13}C]serine and its metabolites are not detected. The intense peak near 160 ppm is due to labeled tyrosine residues.

appears at 28 ppm (Figure 3, bottom right). The intensity of this peak decreases when IPP was bound at the α-subunit site (Figure 3, top right), while a peak at 123 ppm increases in intensity (Figure 3, top left). Both peaks are missing in spectra obtained with unlabeled serine plus IPP (Figure 3, middle), or when neither serine nor IPP was present (data not shown). Solution-state ^{13}C NMR spectra of the mother liquor in contact with Trp-S crystals showed labeled serine (63.0 ppm) was converted to labeled pyruvate (29.5 ppm).

Fluorine-19 REDOR NMR. The full-echo ^{19}F REDOR spectrum of the α_2-[^{19}F]F-[^{13}C]Y-β_2 tetramer shows five partially resolved lines for the 13 [^{19}F]F residues (Figure 4b). These lines appear in three groups with relative integrated intensities of 6:6:1. The 100-Hz ^{19}F line width is determined primarily by residual homogeneous coupling to the protons, as determined by the rate of decay of S_o as a function of N_c. Because REDOR dephasing of side bands is different from that of the centerbands (Figure 4a), side-band intensities cannot be ignored or suppressed but must be combined with the center bands, which is accomplished by Fourier transform of a synchronously sampled data subset (Maricq & Waugh, 1979) with noise reduced by zeroing (Figures 4c−e).

Even though the pattern of [^{19}F]F isotropic shifts changes on addition of serine, particularly near 400 Hz (Figure 5,

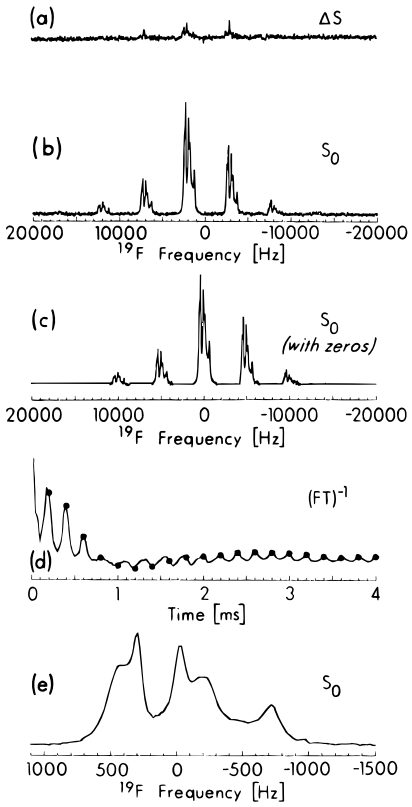


FIGURE 4: 188-MHz ^{19}F REDOR spectra of α_2-[ring-4-^{19}F]-phenylalanine-[phenol-4-^{13}C]tyrosine-β_2 tryptophan synthase after eight cycles of ^{13}C dephasing with magic-angle spinning at 5 kHz. The REDOR difference is shown in (a) and the full echo in (b). Side-band intensities were folded into the center bands quantitatively with no loss in sensitivity by zeroing (c), performing an inverse Fourier transform (d), selecting only the time-domain points corresponding to rotational echoes (d, solid circles), and then performing a final Fourier transform (e). Synchronous-sampling points in (d) do not correspond to echo maxima because of a distribution of ^{19}F isotropic shifts. The reference of the frequency scale is arbitrary.

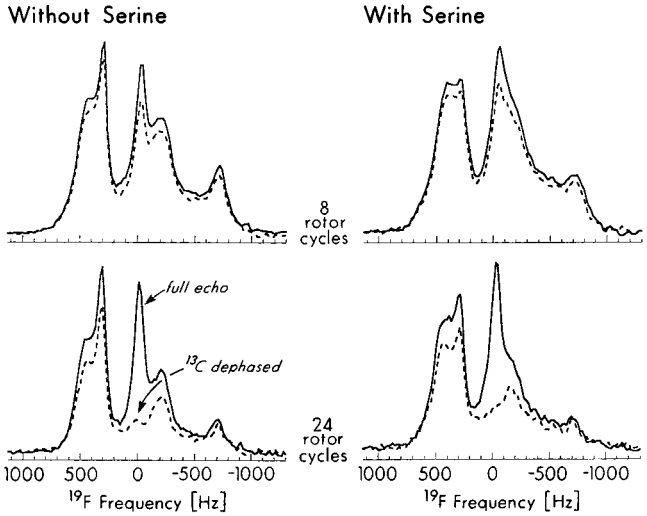


FIGURE 5: 188-MHz ^{19}F REDOR spectra of α_2-[ring-4-^{19}F]-phenylalanine-[phenol-4-^{13}C]tyrosine-β_2 tryptophan synthase with (right) and without (left) added serine after eight rotor cycles (top) and 24 rotor cycles (bottom) of ^{13}C dephasing with magic-angle spinning at 5 kHz. The solid lines show the full-echo spectra and the dotted lines the dephased spectra. The strong dephasing near 0 Hz arises from the three F−Y proximate pairs. The weaker dephasing throughout the spectrum is due primarily to the natural-abundance ^{13}C background. The spectra were obtained by synchronous sampling.

top), there is no significant change near 0 Hz, where full REDOR dephasing for the three proximate F−Y pairs is observed (Figure 5, bottom). To compare qualitatively the predicted values of $\Delta S/S_0$ in Table 1 with the experimental values, we (i) corrected for the incorporation of label for the dephasing nucleus, (ii) accounted for the fact that the full-echo signal had contributions from all 13 [^{19}F]F residues, and (iii) recognized the contributions of the natural-abundance ^{13}C. If there were no natural-abundance ^{13}C, the $\Delta S/S_0$ for the complete ^{19}F-observed REDOR-difference spectrum after 24 rotor cycles of ^{13}C dephasing would arise primarily from the three proximate pairs and would equal $(0.93 + 0.99 + 0.76) \times 0.86/13 = 0.18$ (Table 1). Thus, the area between the solid and dotted lines in Figure 5 (bottom left), which is ΔS, would be 18% of the total. The observed REDOR difference is larger because natural-abundance ^{13}C causes additional dephasing that complicates quantitation. Nevertheless, on a qualitative basis, the ^{19}F-observed relative rates of ^{13}C dephasing show that the three [^{19}F]F which are close to the three [^{13}C]Y (and are therefore associated with rapid dephasing) all have peaks at the same resonant frequency near 0-Hz offset (Figure 5). Because the dephasing for these peaks is nearly the same with and without added serine, the REDOR experiments rule out large relative movements of these residues. We cannot rule out small changes in position. A similar conclusion holds for Trp-S with added serine in the presence of IPP, a specific inhibitor of the α subunit (spectrum not shown). While binding of IPP to the α subunit induces a somewhat different set of ^{19}F chemical shifts in the β subunit with serine added, no qualitatively significant changes in REDOR dephasing rates were observed.

It is possible to account quantitatively for the total dephasing by ^{13}C in the ^{19}F REDOR NMR spectrum by using a model for the lattice to locate the natural-abundance and labeled ^{13}C spins relative to the observed ^{19}F nucleus (McDowell et al., 1996). However, in the present system it is easier to interpret the complementary dephasing by ^{19}F in the ^{13}C REDOR NMR spectrum, as described in the next section.

Carbon-13 REDOR NMR. The ^{13}C REDOR full-echo spectrum of the α_2-[^{19}F]F-[^{13}C]Y-β_2 tetramer with no serine bound shows two partially resolved lines at 155 and 157 ppm due to the 11 [^{13}C]Y residues (Figure 6, bottom). These two lines have relative intensities of 4:7. The other lines in the spectrum are from the natural-abundance ^{13}C in the protein. The three F−Y pairs give rise to a ^{13}C REDOR difference signal at 157 ppm after 24 rotor cycles of ^{19}F dephasing (Figure 6, top). There is no significant natural-abundance background at 157 ppm. With no serine added, $\Delta S/S_0$ for 24 rotor cycles of ^{19}F dephasing is 0.05 (Table 2). A REDOR experiment with 80 rotor cycles of dephasing (spectra not shown) gave an observed $\Delta S/S_0$ of 0.14. Using the distances of Table 1 and taking into account the 37% incorporation of [^{19}F]F, the expected value of $\Delta S/S_0$ for 24 rotor cycles of dephasing is $(0.93 + 0.99 + 0.76) \times 0.37/11 = 0.09$. The expected $\Delta S/S_o$ for 80 rotor cycles of dephasing is 0.12. Both values are in reasonable agreement with the experimental results, taking into consideration the fact that the X-ray distances of Table 1 are not specifically for a fluorinated material and so are only approximate. These $\Delta S/S_0$ values are unchanged by the addition of L-[3-^{13}C]-

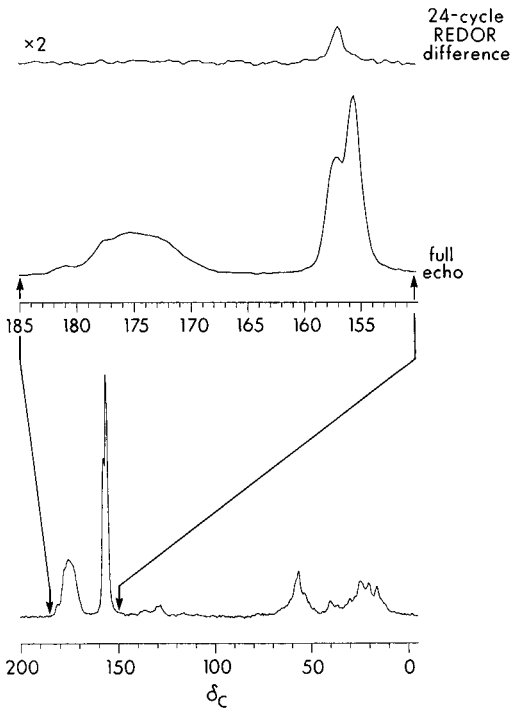


FIGURE 6: 50-MHz ^{13}C REDOR spectra of α_2-[ring-4-^{19}F]phenylalanine-[phenol-4-^{13}C]tyrosine-β_2 tryptophan synthase after 24 rotor cycles of ^{13}C dephasing with 5-kHz magic-angle spinning. The full-echo spectrum is shown at the bottom and the REDOR difference at the top of the figure. Four of the 11 tyrosine residues have ^{13}C aromatic-carbon signals shifted to low field in the full-echo spectrum, and, considering the 37% ^{19}F incorporation for phenylalanine residues, three of these are fully dephased in the REDOR-difference spectrum.

Table 2: Experimental [^{13}C]Y REDOR with ^{19}F Dephasing for α_2-[^{19}F]F-[^{13}C]Y-β_2 Tryptophan Synthase

F280−Y186 + F306−Y279 + F241−Y324	$\Delta S/S_o$ (total)[b] for $N_c = 24$	$\Delta S/S_o$ (total)[b] for $N_c = 80$
without serine (observed)	0.05	0.14
with serine (observed)	0.06	0.13
calculated[a]	0.09	0.12

[a] Using distances from Table 1 and 37% incorporation of ^{19}F-labeled phenylalanine. [b] Estimated experimental error is ± 0.02.

serine, within the estimated experimental error of ±0.02 (Table 2). Error estimates are based on signal-to-noise ratios of the spectra. A $\Delta S/S_o$ for 24 rotor cycles with serine added and with IPP bound at the α-subunit site was 0.06, the same value observed with no IPP bound. None of the ^{13}C REDOR measurements were affected by large-amplitude side-chain motions. Ring flips (if present) do not affect ^{19}F and ^{13}C labels at the ring C-4 position; in addition, the full ^{19}F and ^{13}C chemical-shift tensors (indicated by spinning side-band intensities) rule out large-amplitude reorientational motions for both F and Y ring C_2 axes.

DISCUSSION

Serine Binding. In the absence of IPP inhibitor at the α-subunit binding site, solution-state ^{13}C NMR spectra of the supernatant in equilibrium with crystalline Trp-S complex showed only serine label before the beginning of the solid-state experiments (which ran continuously for more than a month) and mostly pyruvate at the end of these experiments. The pyruvate is formed as a byproduct of slow hydrolysis of the aminoacrylate species when no indole is present at the β site. The ^{13}C label from enzyme-bound serine appears at 28 ppm (Figure 3, bottom right), has intensity corresponding to a single carbon site, and is assigned to the bound intermediate methyl tautomer (**V** or **VI**) of aminoacrylate (Scheme 1). It is highly unlikely that the species giving rise to this resonance is pyruvate or iminopyruvate products because there is no specific affinity of these compounds for Trp-S. If both L-[3-^{13}C]serine and IPP are added to Trp-S, the turnover of serine to pyruvate is greatly reduced (K.S.A., unpublished results), and an enhanced resonance appears in the vinyl region (Figure 3, top left). We attribute this vinyl-carbon peak to aminoacrylate (**IV**). Because the methyl-region peak is reduced in intensity in the presence of IPP (Figure 3, top right), a redistribution of population among aminoacrylate species **IV** and **V** or **VI** apparently has occurred. Such a redistribution is consistent with previous suggestions about the aminoacrylate intermediate (Lane & Kirschner, 1983; Drewe & Dunn, 1985).

In addition to this direct evidence for the effect of serine binding in the ^{13}C NMR spectrum, the ^{19}F isotropic shifts observed in the solid-state experiments after the addition of serine offer indirect evidence. The shifts of the phenylalanine peaks in the 400-Hz region of the ^{19}F NMR spectrum (Figure 5) indicate an altered chemical environment on addition of serine, most likely due to a change in the local electric field associated with nearby charges (de Dios et al., 1993). These changes in ^{19}F chemical shifts are eliminated by repeated washings with serine-free media, which shows that the effect of serine addition on Trp-S in the crystal is reversible despite the apparent disordering on binding. Moreover, the similarity of the ^{13}C REDOR results (with ^{19}F dephasing), before and after serine addition (Figure 6 and Table 2), is only consistent with minor conformational rearrangements along the indole tunnel, or at least that section of the tunnel lined by the F−Y pairs of Figure 2. We estimate that the proximate-pair $^{13}C-^{19}F$ distances are constant to within 1 Å. This estimate is based on the assumption that a large-amplitude conformational rearrangement would necessarily *increase* the short proximate-pair distances of Table 1. Increasing each of the distances by 1 Å would decrease $\Delta S/S_o$ for $N_c = 24$ by half with no change in the $\Delta S/S_o$ for $N_c = 80$. An increase in one or more of the proximate-pair distances by, say, 5 Å, would make detectable decreases in $\Delta S/S_o$ for both $N_c = 24$ and $N_c = 80$. In fact, no decreases are observed (Table 2). We therefore conclude that opening and closing the α−β indole tunnel leaves much of the tunnel conformationally unperturbed and may only require moving a gating side chain by a fraction of an angstrom.

Fluorine-19 NMR. Almost all of the observed 100-Hz ^{19}F line widths of Trp-S (see Figure 4, bottom) arise from residual dipolar coupling to the protons, as established by the 3-ms lifetimes of S_o echo trains. These echo-train lifetimes will need to be increased to 15 ms or more (comparable to the observed lifetimes of the echo train for the ^{13}C label) if the full capabilities of ^{19}F-observed distance measurements are to be realized. For example, attempts to measure specific 10-Å $^{19}F-^{31}P$ distances by ^{19}F REDOR with ^{31}P dephasing were stymied by the short lifetime of the ^{19}F echo train. We anticipate increasing proton decoupling fields to 150 kHz for such experiments in the future. Because most of the ^{19}F line width is homogeneous, NMR experiments performed at higher static fields will clearly result in higher resolution, and this will be important in the future to sort

out complicated spectra like those of Trp-S. Anisotropic bulk susceptibility broadening seems not to be an important contributor to line width. Ultimately, we hope to be able to resolve all 13 [^{19}F]F signals of the β subunit by using a 12-T magnet and ^{19}F detection at 470 MHz.

Carbon-13 NMR. As described above, all of our REDOR results on Trp-S are consistent with the F−Y proximate-pair average distance altered by less than 1 Å on serine addition. Assuming that gating of the tunnel involves side-chain motions of about an angstrom, such subtle changes in conformation can, in principle, be detected using a version of ^{13}C REDOR with ^{19}F dephasing. With ^{19}F dephasing, no background correction is necessary for the ^{13}C-observed REDOR difference signal. In addition, the three F−Y proximate pairs give rise to a full-echo signal that is clearly resolved in the ^{13}C NMR spectrum (Figure 6, top). The dephasing behavior of this signal for N_c varying from 24 to 72 rotor cycles, in increments of 8 rotor cycles, should distinguish between different *distributions* of distances having the same average (Klug et al., 1995). In other words, multiple dipolar couplings might be extracted from a REDOR spectrum having a single isotropic chemical shift, just as multiple quadrupolar splittings are extracted from massively deuterated lipids by de-Pake-ing (Davis et al., 1976). Unfortunately, the sensitivity demands of this procedure put a premium on the incorporation of ^{19}F so that this determination cannot be made successfully on our present double-labeled Trp-S sample. However, we hope to improve on the current level of 37% ^{19}F incorporation by using *E. coli* auxotrophic for phenylalanine. This work is currently in progress.

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