^1H–^{15}N Heteronuclear NMR Studies of *Escherichia coli* Thioredoxin in Samples Isotopically Labeled by Residue Type†

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ABSTRACT: Ten samples of *Escherichia coli* thioredoxin were individually isotopically enriched by residue type via growth of an appropriate auxotrophic strain on media supplemented with one ^2H,^{15}N-enriched amino acid. ^1H observe-heteronuclear decoupling experiments were conducted on these samples making use of the 95-Hz ^1H–^{15}N amide J_1 coupling. Subtraction of near-resonance from off-resonance ^{15}N decoupled spectra generated difference patterns corresponding only to protons directly bonded to ^{15}N nuclei. For the ten different enriched residue types observed to date, every labeled position (60) has been observed as a resolved resonance. The spectral dispersion in both the ^1H and the ^{15}N dimensions was roughly 1500 Hz (at 500-MHz field strength) with rather little apparent dependence on residue type. With the exception of the glycine-enriched sample, the range of the J_1 coupling constants was not much greater than the precision of the measurements (1.5–2.0 Hz). However, for the glycine residues the J_1 amide coupling values varied over a range of 10 Hz.

The difficulties associated with the observation of individual NMR resonances, both for assignment and for measurement of static and dynamic properties, have limited the application of this powerful technique in the study of proteins. The two-dimensional techniques developed by Ernst (Aue et al., 1976a,b) and applied by Ernst and Wüthrich (Nagayama et al., 1977; Nagayama et al., 1979; Kumar et al., 1980) to the study of protein spectra have greatly increased the ability to observe individual resonances. However, their application to proteins containing ^1H, ^{13}C, and ^{15}N at natural abundance levels has several limitations. The spin complexity and relaxation behavior of perprotio samples have limited the size of the proteins to which these 2D techniques have been successfully applied to date. In addition to low natural abundance, the intrinsic insensitivity of the ^{13}C and ^{15}N nuclei render heteronuclear or non-proton homonuclear experiments highly time consuming at best, limiting 2D experiments almost exclusively to the proton.

We have chosen a complementary approach to these problems by developing a versatile isotopic labeling system for the production of proteins enriched by residue type with ^2H, ^{13}C, and/or ^{15}N. Procedures for the initial synthesis of appropriately labeled precursor amino acids have been described previously (LeMaster & Cronan, 1982; LeMaster & Richards, 1982a,b). In this paper we present NMR assignment experiments utilizing *Escherichia coli* thioredoxin, which we hope will become a useful model system for the study of protein dynamics. ^1H–^{15}N heteronuclear decoupling experiments in which individual amide proton resonances are observed through the spin interaction with the directly bonded ^{15}N nucleus are described, allowing observation and assignment of amide protons (and nitrogens) by residue type. For this purpose the protein samples were enriched with ^{15}N in the amide position of a particular residue type. Classical heteronuclear decoupling techniques were employed since the instruments used in this study were not, at the time, equipped for heteronuclear 2D detection. Conceptually similar experiments have recently been conducted on T4 lysozyme (Griffey et al., 1985) as well as on RNA molecules in order to observe imino proton resonances both by 2D and by 1D techniques as described here (Bax et al., 1983; Roy et al., 1984; Kime, 1984). Elsewhere, we have demonstrated that specific residue assignments can be obtained by observing spin interaction across the peptide linkage between the carbonyl carbon and amide protons of the following residue (LeMaster & Richards, 1985). These experiments are similar to those successfully applied to oligopeptides (Llinas et al., 1977; Okhanov et al., 1980).

Thioredoxin is a disulfide–dithiol couple redox enzyme that was first implicated in the reduction of inorganic sulfate in yeast (Black et al., 1960). It was later found to donate reduction equivalents to the ribonucleotide reductase in *E. coli* (Laurent et al., 1964). Holmgren (1981) has conducted a number of biological and physical studies of this protein. Thioredoxin has also been found to serve as a subunit in the T7 phage DNA polymerase (Mark & Richardson, 1976). More recently, Buchanan and co-workers have found that thioredoxin plays an important role in light activation of various photosynthetic enzymes in chloroplasts (Buchanan, 1981).

Although thioredoxin has proven to be a ubiquitous protein with various interesting biological functions, it is the physical properties of this protein that make it appealing for our present use. The *E. coli* enzyme is small (108 residues) (Holmgren, 1968) and is both heat (T_m = 85 °C at pH 7.0) and pH (2.0–11.5) stable (Reutimann et al., 1981). Unlike a number of the other well-studied stable small proteins, thioredoxin is not stabilized by a network of disulfide bonds. The sole disulfide is that of the active site, which spans a β turn on the surface of the molecule (Holmgren et al., 1975). The stability of the reduced form of the enzyme is somewhat less, having a calorimetric T_m of 73 °C at pH 7.0 (D. M. LeMaster and J. M. Sturtevant, unpublished observations). Furthermore, the folding kinetics of the reduced form differ significantly from those of the oxidized form (Kelley & Stellwagen, 1984). The contrast between the physical properties of these two biologically relevant states of the molecule is one of the ap-

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pealing features of this system. Crucial to the practicality of the labeling experiments to be described, the *E. coli* thioredoxin gene has been cloned (Lunn et al., 1984). The plasmid pBHK8 in the *E. coli* DL30 background produces thioredoxin as roughly 1–2% of total cell protein.

The data from 10 different thioredoxin samples enriched with ^{15}N by residue type are reported. In each case every labeled position has been observed as a resolved resonance. To date, we have a total of 60 partially assigned main-chain amide proton resonances out of a total of 102 for the whole protein, whose molecular weight is 11 675.

Materials and Methods

^{15}N- (95%) and ^{2}H- (97%) enriched bulk protein was biosynthetically produced by growth of bacterial strain MRE600, on [$^{2}H_4$]succinate (LeMaster & Richards, 1982a) and $(^{15}NH_4)_2SO_4$ (Mound Laboratories) in a $^{2}H_2O$-containing medium. The protein was hydrolyzed, and the constituent amino acids were separated by ion-exchange chromatography as described elsewhere (LeMaster & Richards, 1982a). These amino acids contained ^{15}N in all nitrogen positions and ^{2}H in all carbon-bound hydrogens. All nitrogen- and oxygen-bound exchangeable deuterons were replaced with protons during the hydrolysis and workup. Labeled *E. coli* thioredoxin was produced by growth of a polyauxotrophic K-12 strain harboring a TrxA-containing plasmid on a defined medium with one amino acid type enriched with ^{15}N and ^{2}H.

Auxotrophic Strain Construction. The polyauxotrophic strain DL30 bears the genotype F–, proA, argE, his, metB, thr-25, serA, glyA(Tn5), srl(Tn10), recA, ilvE12, tyrB507, aspC13, lacYl, galK2, ara-14, xyl-5, mtl-1, str-31, tsx-33, supE44, recB21, recC22, sbcB15, hsdS, lambda–. This strain was constructed by utilizing the glutamate-dependent general amino acid transaminase deficient strain DG30 as parent (Gelfand & Steinberg, 1977). Most of the genetic markers are derived from this strain, and the markers listed after aspC13 have not been verified in the final construction. The strain DG30 requires eight different amino acids. The additional auxotrophies were introduced as follows: A rifampicin-resistant derivative of the metB-containing strain 1005 (kindly provided by David Clark) was used as a source of P1 phage lysates. The rifampicin resistance was transduced into DG30, and the recombinants were screened for the introduction of a methionine requirement from the linked metB gene. This strain was crossed with a cycloserine-resistant derivative of PC0950 (obtained from the Coli Genetic Stock Center) selecting for cycloserine resistance and screening for threonine auxotrophy. The lysA(Tn5) marker of CBK140 (kindly obtained through K. Brooks Low) was transduced in, and then subsequently, the lysine auxotrophy was selected against during transduction from MA197 (obtained from the Coli Genetic Stock Center). The transformants were screened for serine auxotrophy. The last amino acid marker was introduced by transduction of the glyA(Tn5) from CBK081 (kindly provided by K. Brooks Low). The recA mutation was then introduced by transduction of srlA(Tn10) from MC136 followed by screening for increased UV sensitivity. The plasmid pBHK8 (Lunn et al., 1984) was obtained from Vincent Pigiet. The plasmid was transformed into DL30 for the labeling experiments.

Growth Medium. Stationary cultures of DL30 were grown in the following medium (amounts per 12 L): alanine (6.0 g), arginine hydrochloride (7.0 g), aspartic acid (4.8 g), cystine (0.4 g), glutamic acid (8.0 g), glutamine (4.0 g), glycine (6.5 g), histidine (0.7 g), isoleucine (2.8 g), leucine (2.8 g), lysine hydrochloride (5.0 g), methionine (1.1 g), phenylalanine (1.6 g), proline (1.2 g), serine (25.0 g), threonine (2.8 g), tyrosine (2.0 g), valine (2.8 g), adenine (6.0 g), guanosine (8.0 g), thymine (2.0 g), uracil (6.0 g), sodium acetate (18.0 g), succinic acid (18.0 g), ammonium chloride (9.0 g), sodium hydroxide (10.2 g), and dibasic potassium phosphate (126.0 g). All amino acids were reagent-grade L enantiomer samples obtained from Sigma. After autoclaving, the following solutions were added: glucose (120 g) as a roughly 30% solution, $MgSO_4 \cdot 7H_2O$ (3.0 g), $FeSO_4$ (50 mg), 0.1 mL of concentrated H_2SO_4 in 100 mL of H_2O, and 1.2 g of ampicillin titrated to pH 7.0 with NH_4OH in 100 mL of H_2O.

In this medium DL30 grows with a doubling time of roughly 60 min to a stationary density of 7–8 g wet weight per liter in a MF-114 New Brunswick fermentor. The strain is sensitive to room light so that the fermentor jar is wrapped in aluminum foil during growth. When DL30 was grown for a labeling experiment, one amino acid component was removed from the medium list and replaced with a ^{15}N-enriched amino acid sample in growth-limiting amounts.

For most cases that we have studied (aspartic acid, histidine, isoleucine, leucine, methionine, phenylalanine, proline, tyrosine, and valine), the amount of exogenous amino acid required to produce a given amount of cells corresponds to 50–100% excess over the amount of the amino acid obtained from bulk protein (LeMaster & Richards, 1982a). Put somewhat differently, this production and incorporation system is 50–66% efficient for these amino acids when averaged over all proteins of the cell. For the amino acids glycine and arginine, these values are closer to 40%. The case of serine is quite exceptional. The incorporation efficiency is less than 10% so that on a molar basis roughly 10 times as much serine is required than for most other amino acids. For both the [^{15}N]tyrosine and [^{15}N]isoleucine samples described below, 0.5 g of each amino acid was required to produce roughly 60 g wet weight of labeled cells.

Protein Purification. In a typical purification, 60 g wet weight of cells were suspended in ice in 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 8.5, with 2 mM $MgCl_2$ and 2 mM $CaCl_2$ to a total volume of 150 mL. The cells were lysed with a Branson sonifier, Model 185, and the extract was treated with roughly 1 mg of pancreatic DNase at room temperature for 20 min. The cell debris was pelleted at 20 000 rpm for 15 min in an SS34 rotor, and the pellets were resuspended and repelleted in 0.5 volume of Tris buffer. The two supernatants were combined and dialyzed at 4 °C for 3–4 h against 25 mM NH_4HCO_3. This step serves both to desalt and to reduce the viscosity of the concentrated solution described below. The sample was then lyophilized and resuspended in 35 mL of 25 mM NH_4HCO_3, spun at 20 000 rpm for 10 min to remove insoluble debris, and loaded onto a 2.5 × 100 cm column of Ultragel AcA54 equilibrated against the same buffer (Lunn et al., 1984). The column was run at approximately $^{2}/_{3}$ mL/min, and the fractions were analyzed by spotting 5 μL of sample on Whatman No. 1 filter paper and staining briefly with a standard Coomassie blue solution. The thioredoxin peak emerges at approximately $^{2}/_{3}$ column volume, well removed from most other protein contaminants that elute near the void volume. The fractions were pooled, loaded onto a 6-mL bed volume column of DE-52 equilibrated with 25 mM NH_4HCO_3, and washed with 15 mL of 150 mM NH_4HCO_3. The thioredoxin sample is then removed with 15–20 mL of 300 mM NH_4HCO_3. This column removes a few minor protein contaminants as well as small nucleic acid molecules that come off the sizing column. It also serves to concentrate the sample so that a final lyophilization in a silanized 100-mL conical

centrifuge tube is suitable for resuspension of the protein sample in 300–400 μL. As examples, 49 mg of [^{15}N]tyrosine-enriched and 66 mg of [^{15}N]isoleucine-enriched thioredoxin were isolated by the above protocol for the experiments described in this paper.

NMR Spectroscopy. The ^1H–^{15}N heteronuclear decoupled spectra were collected on the Nicolet 470 at the Purdue University Biochemical Magnetic Resonance Laboratory and the Bruker WM500 at the Chemical Instrumentation Center at Yale University. Work at the Purdue facility utilized an 8-mm ^{15}N detection probe with which the ^1H spectra were observed through the decoupling coil. Continuous-wave ^{15}N decoupling was accomplished with a PTS 160 synthesizer under direct operator control. For the later work with the Bruker instrument, a 5-mm ^1H detection probe was used in which the heteronuclear decoupling coil was tuned to the ^{15}N frequency. ^{15}N decoupling was carried out under computer control.

The ^{15}N-enriched amino acid samples were prepared with deuterium in all nonexchangeable hydrogen positions as a means of eliminating the H^{α}–H amide coupling interaction. All protein samples described herein (at concentrations of between 1 and 5 mM) were dialyzed against 200 mM NaCl, pH 3.4, in which the protein served as buffer. One to two percent final concentration of ^2H_2O and approximately 0.5 mM (trimethylsilyl)propanesulfonate were added. The water resonance was suppressed with the 45°–τ–45° pulse sequence described by Kime & Moore (1983). In all cases an ^{15}N decoupling field of 100 Hz was used. Chemical shifts were measured against external 1 M $^{15}NH_4Cl$ in 85% D_2O, 5% H_2O, and 10% HCl (Wilmad reference standard WGN-01) and then referred to nitromethane, assuming the NH_4Cl standard shift was 352.5 ppm (Witanowski et al., 1977).

Results

For each protein sample, we collected a series of ^1H spectra each with the continuous-wave ^{15}N decoupling at a different frequency. The ^{15}N frequency was stepped across the ^{15}N amide spectral region in increments of either 50 or 100 Hz. In addition, spectra were collected in which the ^{15}N frequency was moved roughly 2000 Hz off resonance. Difference spectra were generated between the near- and off-resonance data sets. All resonances should cancel out except for those corresponding to protons directly bound to ^{15}N atoms.

Figure 1 illustrates the pattern obtained for [^{15}N]tyrosine-enriched thioredoxin. First, it is clear that as the ^{15}N frequency is varied in 50-Hz increments we approach the ^{15}N resonance frequencies for both tyrosine amide nitrogens. The ^{15}N decoupling decreases as we move past the resonance condition. As is evident in the figure, the inverted multiplet pattern changes its appearance as a function of the distance from the resonance frequency. Since the off-resonance decoupled spectra appear the same in each case, the change is due to the differences in the near-resonance spectra. When exactly on resonances, we obtain the simplest pattern; the central resonance collapses to a singlet which is subtracted from the constant doublet pattern arising from the normal 95-Hz ^1H–^{15}N coupling. As we move slightly off resonance, the decoupling effect is no longer complete and we obtain a doublet pattern with a reduced coupling constant rather than a singlet. The magnitude of this J_r is dependent on the frequency difference away from exact resonance as well as the decoupling field strength. Hence, if the decoupling field strength is determined independently (γ_{H2} = 100 Hz), the residual coupling gives an accurate measurement of the resonance frequency. Given the line width of the amide reso-

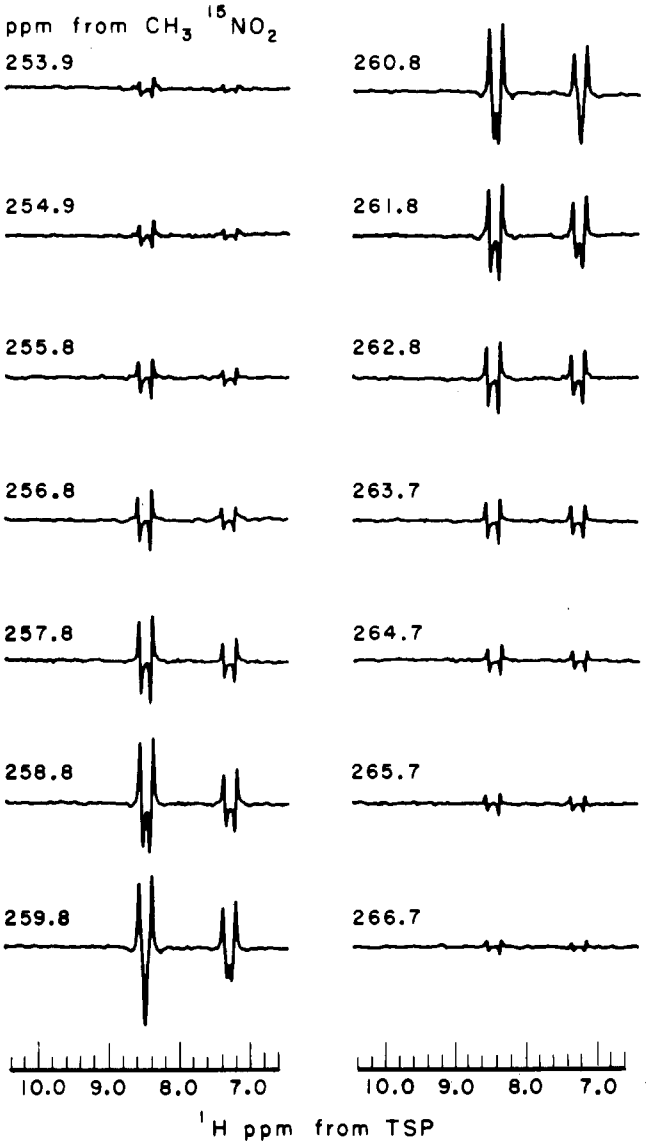

FIGURE 1: ^1H–^{15}N decoupling difference spectra of [^{15}N]tyrosine-enriched *E. coli* thioredoxin. The ^1H amide spectral region was observed at 500 MHz with single-frequency ^{15}N decoupling. A series of spectra were collected, varying the ^{15}N decoupling frequency in 50-Hz increments across the ^{15}N amide spectral region. These spectra were each subtracted from spectra in which the ^{15}N decoupler had been shifted approximately 2000 Hz off resonance. The decoupling field was 100 Hz. Water suppression was achieved with a 45°–τ–45° sequence with the carrier frequency downfield of the water resonance.

nances and the digital resolution used, it is doubtful if the precision of most of our ^{15}N frequency measurements is better than ±20 Hz (0.4 ppm). However, if further experiments are warranted, more accurate determinations are clearly feasible.

One more point to note in the ^{15}N tyrosine spectra is that the intensity of the resonance at 7.34 ppm is less than that at 8.53 ppm. This is a manifestation of the roughly sinusoidal excitation envelope resulting from the water-suppression pulse sequence. As with all such sequences that create a power null at the water resonance, the spectral intensity of resonances decreases as one approaches the water frequency. However, we have readily observed amide resonances upfield as far as 6.7 ppm, and we are confident that resonances could be observed considerably closer to the water resonance.

A similar data set for isoleucine-enriched thioredoxin is presented in Figure 2, which serves to illustrate the degree of spectral resolution offered by this approach. In these spectra seven resonances are observed as well-resolved difference

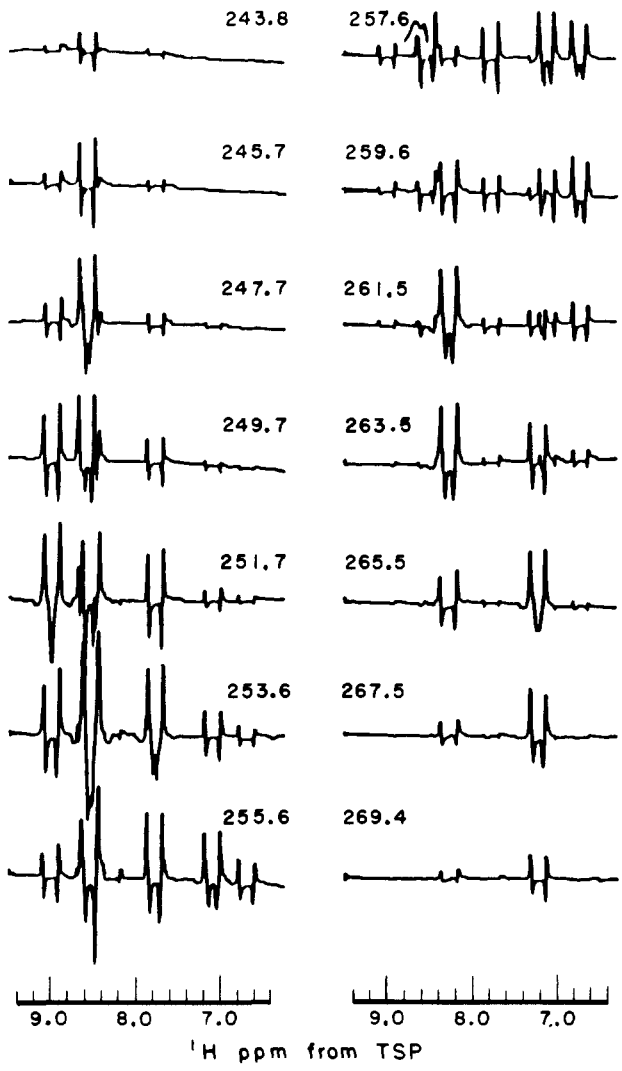

FIGURE 2: ^{1}H–^{15}N decoupling difference spectra of [^{15}N]isoleucine-enriched *E. coli* thioredoxin. Spectra were collected as described in the legend of Figure 1 except that the ^{15}N decoupling field was varied in 100-Hz increments. In the 257.6 ppm difference spectra, the peak at 8.65 ppm is plotted on an expanded scale to demonstrate the two resonance components.

patterns. However, the difference pattern centered at 8.55 ppm ^{1}H and 253.7 ppm ^{15}N is in fact due to two severely overlapped resonances. This example is the most extreme case of spectral overlap we have observed to date. Nevertheless various components of the multiplet pattern are in fact resolved into two peaks as illustrated in the 257.6 ppm ^{15}N-decoupled spectrum of Figure 2. We believe this experiment illustrates the value of using deuterated samples as a means of eliminating the broadening of the amide proton resonance due to the vicinal coupling. It seems quite unlikely that these two resonances would have been resolved if an additional 7–10-Hz line broadening were present.

Not only for isoleucine but for the various other types of residues studied is there a considerable spectral heterogeneity in both the ^{1}H and ^{15}N dimensions, and the chemical shifts of the coupled pairs of ^{1}H and ^{15}N amide resonances show a surprising degree of independence. This is most clearly illustrated in Figure 3 in which a two-dimensional plot of the observed pairs is given, each position represented by the one-letter code for the amino acid type. On close examination, it is clear that there is an overall bias to lie along a diagonal from the upper left to lower right of the diagram. This line corresponds to a coupling of upfield or downfield shift effects

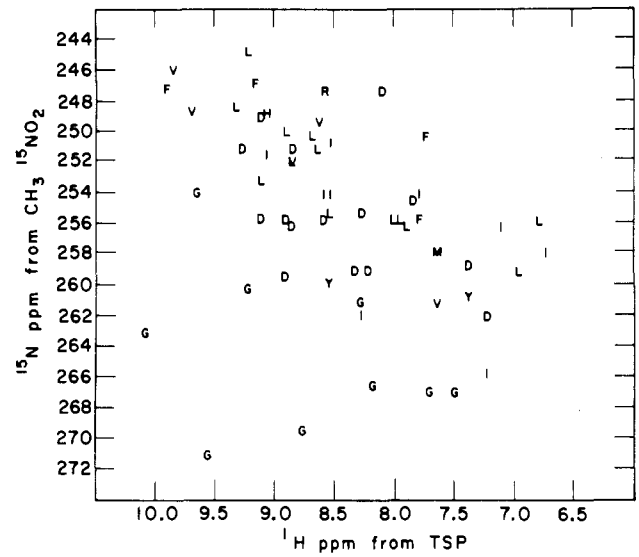

FIGURE 3: ^{1}H and ^{15}N chemical shifts of coupled peptide resonances in *E. coli* thioredoxin. Single-letter amino acid codes are placed at ^{1}H and ^{15}N resonance positions corresponding to the coupled nuclei. Sixty pairs of resonances from ten amino acid types have been observed to date.

between the two nuclei. However, the bias is weak. For 2D experiments, these nuclei will have nearly the spectral dispersion anticipated for two independent chemical shifts.

This spectral dispersion is in marked contrast to what has been found in the imino nitrogen studies of small RNA molecules. In yeast tRNA (Roy et al., 1984) and *E. coli* 5S RNA (Kime, 1984) the total spectral spread of the guanosine N1 in GC base pairs as well as the uracil N3 in AU base pairs is only about 3–4 ppm, roughly one-fourth of what is seen for the protein ^{15}N amide resonances. The reasons for the strikingly different chemical shift behavior is unclear.

Table I presents the chemical shift data of Figure 3 as well as a selected number of coupling constant measurements. Under the conditions of these experiments the J_1 values are probably accurate to only 1.5–2.0 Hz. However, the following general conclusions can be drawn from these data. The coupling constants show a small dependence on side-chain type. Except for glycine, within a given residue type there appears to be rather little dispersion in the J_1 values. In contrast, glycine residues show a much wider dispersion. One plausible explanation might be that the J_1 value is dependent on the main-chain ϕ dihedral angle for which glycine residues show far greater variation. This result is somewhat disappointing since we had hoped that the magnitude of the coupling constant might reflect variations in hydrogen bonding. Evidence has previously been presented (Pardi et al., 1983) that in protein spectra amide proton shifts correlate with hydrogen-bond lengths. However, there are so many other poorly quantitated contributions to chemical shift that the predictive value of the hydrogen-bonding contribution appears to be severely limited. Although more accurate coupling constant measurements using heteronuclear *J*-resolved experiments over a larger set of spectra are warranted, it appears that the ^{1}H–^{15}N coupling constant may be similarly restricted in its use as a monitor of hydrogen bonding.

Discussion

In this study the majority of a given class of protein nuclei have been observed as individual resonances by utilizing a series of selectively enriched samples. We have found considerable spectral heterogeneity in both the ^{1}H and ^{15}N dimensions for individual residue types. All the enriched samples

Table I: Chemical Shifts (ppm) and Coupling Constants (Hz) of ^1H and ^{15}N Resonances in *E. coli* Thioredoxin

amino acid	^1H shift	^{15}N shift[a]	J_1[b]	amino acid	^1H shift	^{15}N shift[a]	J_1[b]
Arg	8.59	247.0	91.5	Ile	8.28	262.1	93.5
Asx	9.24	251.3			7.77	253.9	92.5
	9.09	248.8	92.5		7.22	265.6	92.5
	9.09	255.9			7.09	256.4	93.5
	8.88	259.8			6.69	258.0	90.5
	8.88	255.9		Leu	9.32	248.3	95.5
	8.81	256.3			9.20	244.7	97.5
	8.81	251.3			9.08	253.3	93.5
	8.54	255.3			8.86	251.3	
	8.34	259.2			8.82	252.1	
	8.26	255.3			8.67	249.9	
	8.23	259.2			8.60	251.3	
	8.11	246.9	92.5		8.50	255.1	
	7.85	254.3			7.99	255.9	
	7.32	258.6			7.96	255.9	
	7.19	262.6			7.87	256.3	
Gly	10.16	263.2	92.5		6.93	259.2	94.5
	9.62	253.7	101.5		6.74	255.8	93.5
	9.56	271.2	91.5	Met	7.63	257.6	94.5
	9.20	260.4	96.5	Phe	9.92	247.0	92.5
	8.76	269.3	93.5		9.15	246.6	94.5
	8.14	261.2			7.76	255.9	90.5
	8.14	266.5	93.5		7.75	250.1	92.5
	7.70	267.1		Tyr	8.53	260.0	90.5
	7.51	267.1			7.34	260.6	91.5
His	9.03	248.6	92.5	Val	9.83	245.8	94.5
Ile	9.00	251.7	93.5		9.71	248.6	93.5
	8.59	248.4	93.5		8.79	252.1	93.5
	8.55	253.7			8.60	249.4	93.5
	8.53	253.7			7.64	261.2	91.5

[a] Precision for most ^{15}N resonance positions is approximately 20 Hz (0.4 ppm). ^{15}N shifts were measured relative to $^{15}NH_4Cl$ and referenced to nitromethane, assuming a $^{15}NH_4Cl$ shift of 352.5 ppm. [b] Precision for the coupling constants is 1.5–2.0 Hz.

so far examined have yielded resolved resonances under a standard set of conditions. Although occasional degeneracies will surely occur, we are confident that the vast majority of the amide resonances will prove resolvable by using selective enrichment for proteins considerably larger than the 108 residue *E. coli* thioredoxin.

Given this potential, some consideration of the practical generality of these experiments appears warranted. The thioredoxin data were collected at pH 3.4. This pH was chosen not only to ensure a slow amide exchange rate but because of the tendency of thioredoxin to aggregate within ±1 pH unit of its isoelectric point of 4.7. Although no detailed study of the amide-exchange kinetics of this protein has yet been conducted, by analogy with others, such as BPTI (Wagner & Wüthrich, 1982), we would expect pH 3.4 to be close to the minimum in the pH dependence of the hydrogen-exchange kinetics. None of the observed resonances show any apparent broadening as might be expected if the exchange rate became comparable to the coupling constant (95 s^{-1}). Model peptide studies show that HX rates are in the neighborhood of 0.01 s^{-1} under the same conditions (Molday & Kallen, 1972). This rate for free peptides would be expected to rise to about 100 s^{-1} in the pH range 7.5–8.0. When combined with the success of others in observing amide resonances at neutral pH in COSY (Hore, 1984; Guittet et al., 1984) as well as double quantum (Prestegard & Scarsdale, 1985) experiments using excitation null techniques for water suppression, this estimate makes it likely that the procedures described in this paper may be useful for the entire amide population at pH values below 7 and certainly below 6.

There are several points to consider as to the selectivity and generality of the enrichment by residue type procedure. The biosynthetic and chromatographic techniques previously described are suitable for producing adequate amounts of all ^{15}N-labeled amino acids except cysteine, asparagine, and glutamine. In no case have we seen evidence for low-level secondary labeling of other residue type that might have been caused by transaminases during the incorporation of the labeled amino acids into thioredoxin. Furthermore, there appears to be little evidence of isotopic dilution of the labeled amide groups. We have not yet carried out these labeling experiments for all the possible residue types. However, we anticipate problems in only a few cases. To our knowledge, no one has isolated a useful alanine auxotroph in *E. coli* (Falkinham, 1977). Furthermore, although strains lacking both glutamate synthetase and glutamate dehydrogenase require glutamate supplementation, glutamate is so central to nitrogen metabolism that it seems likely that much of the glutamate nitrogen would be exchanged during incorporation. In particular, there appears to be a glutamate-alanine transaminase activity for which there presently exists no known mutations (Umbarger, 1978). One unexpected complication arose in the case of threonine. For suitable log growth of the DL30 strain, the concentration of threonine must be greater than 1.5 mM. This is significantly larger than the amount that we would expect to be required for growth to stationary density on the basis of only the threonine content of the bulk protein. Growth yields as a function of threonine concentration below 1.5 mM were decidedly nonlinear; indeed, often no growth at all was observed. Although we have not determined the basis of this effect, a plausible possibility is the competition, through destruction by threonine deaminase (ilvA), for the exogenously added threonine. In all other cases, growth yields follow a linear dependence on amino acid concentration up to stationary growth conditions. This includes serine, cystine, and proline, which are not reported elsewhere in this paper.

With the various qualifications described above, the system represents a versatile means of introducing selective labeling into one or several residue types. It should be applicable to any protein that can be cloned and expressed in *E. coli*. The

^1H–^{15}N heteronuclear decoupling experiments serve to illustrate the spectral simplification made accessible by these labeling techniques. The most obvious use of this particular labeling pattern is the potential application to amide-exchange studies. Each [^{15}N]tyrosine difference spectrum corresponds to two spectra each collected in 5 min. Thus, it is feasible to get high-quality data in a period of time suitable for standard exchange-out experiments. The known shift positions of the amide protons by residue type make it possible to select sets of labeled residues that give rise to resolved resonances. Selective decoupling would allow for sampling of subsets of the total enriched sample, while with nonselective decoupling one could monitor all labeled positions at once.

These samples are suitable for 2D COSY experiments as well. The large one-bond couplings should prove highly beneficial for larger protein molecules. When the coupling constants utilized in a COSY experiment are equal to or smaller than the relaxation rates of the nuclei involved, the cross-peaks are attenuated by mutual cancellation of their antiphase components. This effect is central to the practical limitation of ^1H–^1H COSY to small proteins since usually 5–8-Hz couplings are utilized. Use of larger one-bond couplings such as ^{13}C–^{13}C and ^{13}C–^1H, as well as ^1H–^{15}N, should serve to overcome this problem in the application of two-dimensional techniques to moderate-size proteins.

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