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^{15}N-Labeled P22 c2 repressor for nuclear magnetic resonance studies of protein-DNA interactions

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Abstract. The salmonella phage P22 c2 repressor was produced with 90% ^{15}N isotope labeling of all leucines, using the expression system *E. coli* W3110 *lac* I^Q/pTP 125. The *N*-terminal DNA-binding domain 1-76 was obtained by chymotrypsin cleavage. Its characterization by biochemical techniques, mass spectrometry, and one- and two-dimensional nuclear magnetic resonance (NMR) showed that highly residue-selective isotope labeling was achieved with the minimal growth medium used. The ability to obtain such isotope labeling opens new avenues for NMR studies of protein-DNA interactions in the P22 operator system.

Key words: Isotope labeling, nuclear magnetic resonance, protein conformation, protein-DNA interactions, P22 c2 repressor

Introduction

This paper reports initial results from a project on investigations of protein-DNA interactions involving the c2 repressor system of salmonella phage P22. The principal experimental technique used is nuclear magnetic resonance (NMR) in solution, and it is planned to investigate complexes formed between the DNA-binding region 1-76 of the c2 repressor and synthetic oligonucleotide analogs of the operator region. Two critical requirements for such studies are that the NMR spectral resolution is sufficient for distinguishing between resonance lines originating from the protein and the DNA, respectively, and that sequence-specific resonance assignments are available for both components (Wüthrich et al. 1982; Wüthrich 1986). To improve the spectral resolution for certain NMR experiments with the protein-DNA complexes (e.g., Bax et al. 1983; Brühwiler and Wagner 1986; Griffey et al. 1985b; Roy et al. 1984; Weiss et al. 1986; Wörgötter et al. 1986) we prepare analogs of the c2 repressor with isotope labeling by residue type. These isotope labels also represent a most useful complementation of the sequential assignment procedures (Billeter et al. 1982; Wagner and Wüthrich 1982) for obtaining sequence-specific ^{1}H NMR assignments of the c2 repressor 1-76 (Wüthrich 1986). In the following we describe the preparation of c2 repressor 1-76 containing [2-^{15}N]-leucine, and the characterization of the labeled protein with biochemical techniques, mass spectrometry, and NMR.

Results

P22 c2 repressor was obtained from the high-level expression system of *E. coli* W3110 *lac* I^Q/pTP 125, in which about 15% of the total cell protein produced is c2 repressor (Anda et al. 1983). Selective ^{15}N labeling of all leucyl residues was achieved by first growing a culture of the *E. coli* cells with aeration in the complex LB-broth medium (Anda et al. 1983). Before the c2 repressor biosynthesis was induced with isopropyl-1-thio-β-galacto-pyranoside (IPTG), the medium was changed to a defined minimal medium containing [2-^{15}N]-leucine (for details see Materials and Methods). The resulting [2-^{15}N]-leucyl repressor was then cleaved with chymotrypsin to obtain the *N*-terminal fragment 1-76, which was previously shown to retain the specific DNA binding properties of the intact repressor (Anda et al. 1983). All further characterization was done for c2 repressor 1-76.

The appearance of a single sharp protein band in SDS-gel electrophoresis and the agreement of the

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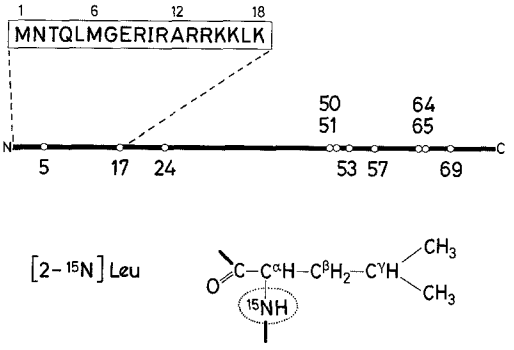

Fig. 1. Scheme presenting the sequence positions of the 10 leucines in c2 repressor 1-76. For the *N*-terminal octadecapeptide segment, the complete sequence is indicated with the one-letter symbols for amino acid residues. The structure of ^{15}N-labeled Leu is indicated at the bottom

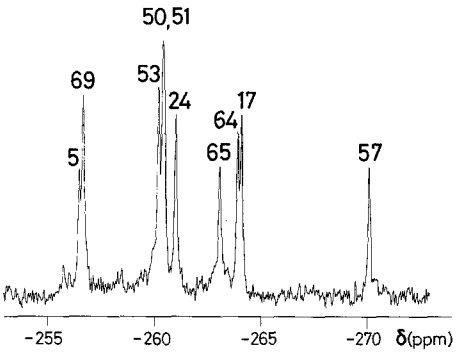

Fig. 2. One-dimensional ^{15}N NMR spectrum of a 6 m*M* aqueous solution of P22 c2 repressor 1-76 at pH 4.8 and 20 °C, recorded with the refocused INEPT technique using broadband decoupling during detection. The resonance lines are identified with the sequence locations of the 10 leucyl residues in c2 repressor 1-76

amino acid analysis with the published data (Sauer et al. 1981) provided an initial indication for a predominantly homogeneous preparation of c2 repressor 1-76. Further analysis by *N*-terminal sequence determination using an automated sequencer revealed the following sequence heterogeneity in the repressor fragment: Only approximately 80% of the polypeptide chains present in the solution had methionine as the N-terminal amino acid, which coincides with the previously reported sequence (Sauer et al. 1981). The remaining 20% of the protein chains started with Asn (position 2 in Fig. 1). No formylated methionine could be detected at the *N*-terminus, and there were no further heterogeneities in the segment 1-18.

The levels of ^{15}N incorporation in c2 repressor 1-76 were investigated by mass spectrometry of hydrolysates isolated after different, individual steps of the Edman degradation. The purified PTH-amino-acid fractions from degradation cycles 3 (Thr), 5 (Leu), 7 (Gly) and 17 (Leu) showed 93% and 89% ^{15}N-enrichment for Leu at the sequence positions 5 and 17, respectively, and a natural abundance ^{15}N-level for Thr 3 and Gly 7. An average ^{15}N-enrichment of 85% for the leucines at positions 24, 50, 51, 53, 57, 64, 65 and 69 (Fig. 1) was determined from the PTH-Leu fraction isolated from the hydrolysate of the peptide fragment 19-76.

The following NMR experiments confirmed the specific ^{15}N labeling of Leu in c2 repressor 1-76 and further resulted in the determination of the chemical shifts of the amide ^{15}N, the amide protons, and the C^{α} protons in all 10 leucyl residues (Fig. 1, Table 1). The sequence-specific assignments of the leucyl NMR lines used in the presentation of Table 1 and Figs. 2 and 3 were obtained by sequential assignment procedures (H. Senn, G. Otting and K. Wüthrich, to be published).

Table 1. ^{15}N, amide proton and C^{α} proton chemical shifts of the 10 leucyl residues in P22 c2 repressor 1-76, at 20 °C

Residue[a]	Chemical shift (ppm)		
	NH	$C^{\alpha}H$	^{15}NH
Leu 5	8.63	4.56	−256.3
Leu 17	7.62	4.23	−264.1
Leu 24	8.13	4.31	−261.0
Leu 50	8.40	3.81	−260.5
Leu 51	7.70	4.07	−260.5
Leu 53	9.10	3.96	−260.1
Leu 57	8.21	4.37	−270.1
Leu 64	7.08	3.49	−263.9
Leu 65	8.52	4.22	−263.0
Leu 69	8.56	4.45	−256.7

[a] Sequence-specific assignments from H. Senn, G. Otting and K. Wüthrich (to be published)

Figure 2 shows a one-dimensional ^{1}H-decoupled ^{15}N-spectrum of c2 repressor 1-76 obtained by the refocused INEPT technique (Burum and Ernst 1980). In an experiment of this kind, each ^{15}N position gives rise to a single, sharp line. 9 resolved lines can readily be distinguished in Fig. 2. Integration showed equal intensity for all these resonances, except that the line at −260.5 ppm has two-fold intensity relative to the others. Thus, this spectrum can account for the 10 [2-^{15}N]-leucyl residues also observed by mass spectroscopy.

Figure 3 shows two regions from a two-dimensional $^{15}N-^{1}H$ heteronuclear relayed coherence transfer experiment of c2 repressor 1-76 recorded with proton observation (Brühwiler and Wagner 1986). This spectrum contains exclusively resonance lines originating from the ^{15}N-labeled leucyl residues, since all peaks corresponding to positions with natural abundance of ^{15}N are too weak to be seen.

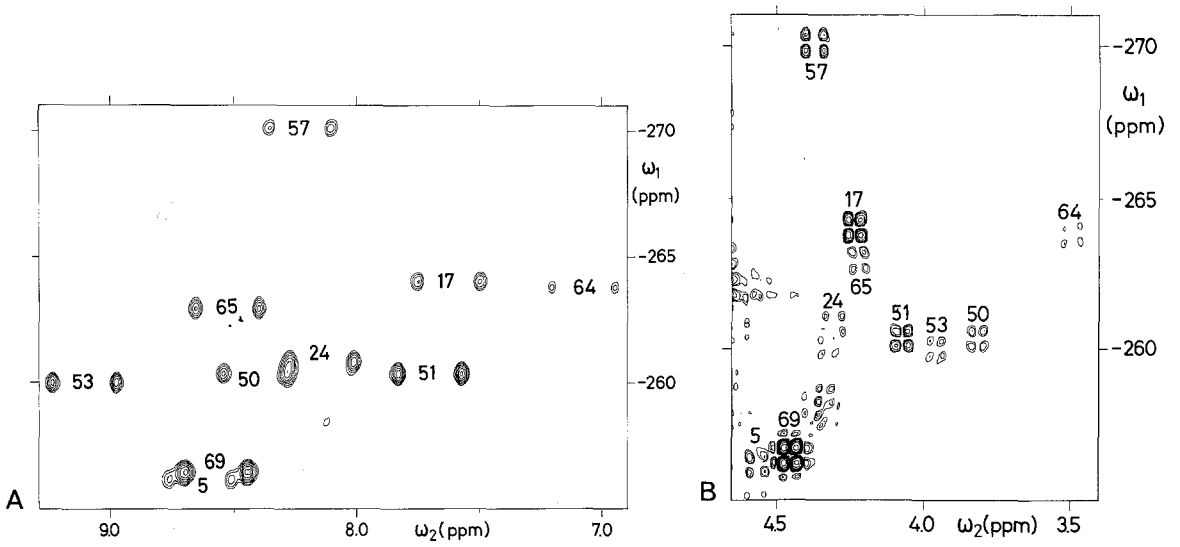

Fig. 3 A and B. Two-dimensional proton-detected heteronuclear correlation experiment with relay pulse. Same sample and conditions as in Fig. 2. **A** Correlations between ^{15}N and the directly bound protons. The line shape is in-phase absorptive with $^{15}\underline{N}-^{1}\underline{H}$ coupling constants of 91.5 ± 2 Hz. **B** Relayed peaks between ^{15}N and $C^{\alpha}\underline{H}$. The multiplet pattern is antiphase with respect to the $N\underline{H}-C^{\alpha}\underline{H}$ proton-proton coupling in both dimensions. In both spectra the cross peaks are identified with the sequence locations of the leucines in c2 repressor 1-76. Additional unidentified peaks in (**B**) resulted from t_1-noise at the water position, and from the presence of some impurities due to slow degradation of the protein during the measurement

Along the vertical ω_1 axis there are peaks at the chemical shift positions of the ^{15}N lines seen in Fig. 2. Along the horizontal ω_2 axis there are the direct cross peaks with the amide protons (region A), which are split into two components separated by the spin-spin coupling constants $^{1}J_{15_{NH}}$ along ω_2, and the relayed cross peaks with the α-protons of the same residues (region B). Starting from the strong ^{15}N signals in Fig. 2, the cross peaks for all 10 leucines can readily be identified at the chemical shift positions listed in Table 1. (The much smaller intensity of the $^{15}N-H$ cross peaks for Leu 64, as well as the origin of the weak signals which do not coincide with any of the ^{15}N signals in Fig. 2 are as yet not explained.) For all 10 leucines the one-bond $^{15}N-^{1}H$ coupling constants was found to be within the range 91.5 ± 2 Hz.

Materials and methods

Biological material, incorporation of [2−^{15}N]-leucine and isolation of c2 repressor 1-76

The plasmid expression system of *E. coli* w3110 *lac* I^{Q}/pTP 125 (Anda et al. 1983) was given to us by Dr. R. Sauer (Department of Biology, MIT). The *E. coli* cells were grown with aeration at 37 °C in 7 l of LB-broth (10 g/l Bacto Trypton; 5g/l yeast extract; 10 g/l NaCl; pH 7.5) plus 100 µg/ml ampicillin to an A_{600} of approx. 1.0. The cells were then harvested by centrifugation (4,000 rpm, 5 min) and washed with 2 l of physiological NaCl solution (0.9 g NaCl/l). The washed cell pellets were then inoculated in a 10 l fermenter containing the following components per liter: 400 mg Ala, 400 mg Glu, 400 mg Gln, 400 mg Arg, 250 mg Asp, 100 mg Asn, 50 mg Cystine, 50 mg Cys, 400 mg Gly, 100 mg His, 100 mg Ile, 100 mg Lys, 250 mg Met, 100 mg Pro, 1,600 mg Ser, 100 mg Thr, 50 mg Trp, 100 mg Tyr, 100 mg Val, 50 mg Phe, 125 mg guanosine, 125 mg uracil, 125 mg cytosin, 50 mg thymine, 500 mg $MgSO_4$, 10 mg $CaCl_2$, 5 mg $FeSO_4 \cdot 7H_2O$; 50 mg nicotinic acid, 50 mg thiamin, 0.1 mg biotin; 2 g sodium acetate, 1 g ammonium chloride, 10 g K_2HPO_4, 2 g succinic acid, 5 g glucose. (This medium was adopted with slight changes from Griffey et al. 1985a.) 10 min after inoculation (37 °C, 10 l air/min.) 1,000 mg of 99% enriched [2-^{15}N]-leucine (Stohler) was added to the fermenter. Induction of c2 repressor production was then initiated by addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 10^{-4} *M*. Growth at 37 °C was continued for 3 h. At the end of the fermentation process an A_{600} of 1.7 was reached. The cells were harvested by centrifugation, washed with physiological NaCl solution and recentrifuged to yield 17 g of wet cell paste.

The c2 repressor was isolated following the purification protocol given by Anda et al. (1983). Proteolytic digestion of the repressor was performed in buffer B (50 m*M* Tris-HCl, pH 7.5, 0.1 m*M*

EDTA, 1.4 m*M* 2-mercaptoethanol, 5% glycerol) plus 0.45 *M* NaCl at room temperature for 15 min at an enzyme-to-substrate ratio of 1:200 (w/w). α-Chymotrypsin (Sigma) was used as proteolytic enzyme. The digestion reaction was stopped by the addition of phenylmethylsulfonyl fluoride at 10 times the concentration of enzyme. About 150 mg of protease-treated repressor were diluted by the addition of 2 volumes of buffer B without NaCl and were loaded onto a column (1.5 × 30 cm) of SP (sulfopropyl)-Sephadex (C-25) equilibrated in buffer B plus 0.15 *M* NaCl. Under these conditions, *N*-terminal repressor fragments are retained on the SP-Sephadex column while *C*-terminal fragments and residual intact repressor are found in the flow-through. The SP-Sephadex column was developed with a linear salt gradient from 0.15 to 0.8 *M* NaCl in buffer B. The total gradient volume was 500 ml and the flow rate was 0.2 ml/min. The *N*-terminal repressor fragment eluted as a sharp peak from the SP-Sephadex column and required no further purification as judged by SDS-gel electrophoresis. 8 g of wet cell paste yielded approximately 35 mg of c2 repressor 1-76.

Analytical methods

The purity of the isolated c2 repressor and its proteolytic fragment 1-76 was checked by polyacrylamide gel electrophoresis (15% gel concentration) in the presence of sodium dodecyl sulphate (Weber and Osborn 1969).

Amino acid analysis. 20 µg of protein were hydrolysed in 200 µl of constantly boiling HCl at 110 °C. The samples were analysed on a Biotronic LC 6000 E amino-acid-analyser.

Determination of the N-terminal amino acid sequence. 4 mg of c2 repressor 1-76 were subjected to Edman degradation in an automated Beckman 890 C sequencer. Polybrene (Sigma) served as the carrier (Tarr et al. 1978). PTH-amino acid derivatives were identified by HPLC (Frank and Strubert 1973). PTH-Arg and PTH-Lys were identified on an isocratic HPLC-system on Partisil 5 PAC (Whatmann).

Mass spectrometric analysis: 400 n*M* [2-^{15}N]-leucine enriched repressor 1-76 were sequenced by automated Edman degradation up to sequence position 18. The isolated PTH-amino acid derivatives from the degradation cycles 3, 5, 7 and 17 (Thr, Leu, Gly, and Leu, respectively, see also Fig. 1) were analysed by mass spectrometry. The samples were directly injected (Hitachi RMU 6 M). The uncleaved peptide fragment 19-76 was extracted from the sequencer cup with 80% acetic acid, dried and hydrolysed (6 *N* HCl, 48 h, 110 °C). The hydrolysed amino acids were then converted to the PTH-derivatives and PTH-leucine was isolated by HPLC and subjected to mass spectrometry. (This PTH-leucine pool resulted from the leucyl residues in the sequence positions 24, 50, 51, 53, 57, 64, 65 and 69; see Fig. 1.)

NMR measurements. 25 mg of c2 repressor 1-76 was dissolved in 0.35 ml 25 m*M* phosphate buffer in H_2O, pH 4.8, containing 10% ^2H_2O, 100 m*M* KCl and 2 m*M* NaN_3. The NMR spectra were recorded on a Bruker AM 360 instrument. Chemical shifts are in parts per million (ppm) from internal 3-trimethylsilyl[2,2,3,3,-$^2H_4$] propionate for protons, and from external $C^2H_3{}^{15}NO_2$ in $C^2H_3{}^{14}NO_2$ for nitrogen-15. Refocused ^{15}N-INEPT spectra (Fig. 2) (Burum and Ernst 1980) were recorded with the pulse sequence $(\pi/2)_H-\tau-(\pi)_H(\pi)_N-\tau-(\pi/2)_H(\pi/2)_N-\tau-(\pi)_H(\pi)_N-\tau-{}^{15}$N-acquisition with ^{1}H decoupling. 5,100 scans were accumulated in about 2.7 h with $\tau = 2.77$ ms, which is optimal for polarization transfer via 90 Hz couplings. A 1 Hz line broadening was used for Fourier transformation.

^{1}H-detected two-dimensional heteronuclear shift correlation spectra and relayed coherence transfer spectra (Fig. 3) (Brühwiler and Wagner 1986) were obtained with the pulse sequence $(\pi/2)_H-\tau_0-(\pi)_H(\pi)_N-\tau_0-(\pi/2)_H-$homospoil$-\tau_1-(\pi/2)_H(\pi/2)_N-t_1/2-(\pi)_H-t_1/2-(\pi/2)_N-\tau_0-(\pi)_H(\pi)_N-\tau_0-(\pi/2)_H-{}^1$H-acquisition, with the phase cycle given in Brühwiler and Wagner (1986). 120 t_1-values were accumulated in about 60 h using a maximum t_1-value of 48 ms and a recycle time of 1 s. τ_0 was 2.77 ms. During the relaxation delay the water signal was suppressed by selective irradiation. The delay τ_1 after the homospoil pulse was varied between 2.0 and 3.9 ms in order to average possible coherent artifacts arising from disturbance of the lock system. As can be shown by a product operator analysis (Sørensen et al 1983) the relayed cross peaks have a dispersive line shape in both dimensions when the direct ^{15}N−^{1}H correlation peaks are phased to be absorptive. Therefore two different Fourier transformations were performed for obtaining the spectra (A) and (B), where the zero order phase parameters used for (A) were incremented by 90 degrees in both dimensions in order to obtain the relayed peaks in absorption. Before the Fourier transformation (A) a cosine window was applied along ω_1, and no data weighting was used along $\omega_2 \cdot t_{2,\max}$ was 174 ms. Before the Fourier transformation (B) the data were weighted with sine bells both along ω_1 and $\omega_2 \cdot t_{2,\max}$ was 110 ms. (In both spectral regions

the cross peaks contain unresolved fine structure from $^1H-^1H$ coupling constants which are small compared to 90 Hz for the $^{15}N-^1H$ splittings seen in (A) or the resolved $N\underline{H}-C^{\alpha}\underline{H}$ couplings in (B).)

Discussion

With the studies reported in this paper we have found a system for efficient selective isotope labeling by residue type in the phage P22 c2 repressor, which offers a variety of new possibilities for NMR studies of this protein and its complexes with DNA fragments. In the preparation of c2 repressor 1-76 described in the preceding section, the abundance of approximately 90% ^{15}N in all 10 leucyl residues corresponds to a 250-fold increase over the natural ^{15}N-abundance of 0.37%. Therefore the NMR lines of the labeled residues stand out clearly above the background of the nonlabeled bulk of the protein (Figs. 2 and 3). The important point is that with the procedures used here, no transaminase-catalysed metabolic diffusion of the ^{15}N-labels into other amino acids occurred. This shows that the presence of high levels of all 20 amino acids in the minimal medium used for the repressor expression was sufficient to suppress essentially all the pathways for amino acid biosynthesis, including transamination.

While the presently used minimal medium provided the desired selective labeling of c2 repressor with [2-^{15}N]-leucine, it also caused the appearance of a N-terminal heterogeneity in the protein product (Met 1 was removed in ca. 20% of the protein). This post-translational protein modification, can be blocked by changing the medium, e.g., no *N*-terminal heterogeneity was observed when the c2 repressor was expressed in a complex LB-broth medium. While the occurrence of this modification in our medium is as yet unexplained, it may be added that post-translational *N*-terminal modifications have been observed for other proteins produced in high yield from *E. coli* (Wold 1981), and that they seem to be quite nonspecific.

Figures 2 and 3 provide an initial illustration of the advantages of using ^{15}N labeled repressor for NMR experiments. In addition to direct observation of ^{15}N (Fig. 2) or observation of correlations with ^{15}N (Fig. 3), which might also provide structural information through the ^{15}N chemical shifts and the heteronuclear spin-spin couplings (e.g. Llinás et al. 1976), the presence of ^{15}N may also be used for obtaining simplified ^1H NMR spectra through suitable filtering or editing procedures. Examples are ^{15}N-relayed proton correlation spectroscopy (Neuhaus et al. 1984; Delsuc et al. 1984), and different *X*-filtered two-dimensional ^1H NMR experiments (e.g., Wörgötter et al. 1986). A whole catalogue of such experiments are available to make use of isotope labels in studies of protein conformation as well as for investigations of intermolecular interactions.

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