Application of site-directed mutagenesis in nuclear magnetic resonance spectroscopy

Christopher J. Penington and Gordon S. Rule
Department of Biochemistry, University of Virginia, Charlottesville, Virginia 22908 USA

INTRODUCTION

To solve the structure of a protein by nuclear magnetic resonance (NMR) methods it is necessary to resolve and assign peaks to specific residues in the protein and obtain distance constraints between assigned residues on the protein. For small proteins ($M_r < 20,000$) of known sequence these problems have been elegantly solved by the application of multidimensional NMR techniques. Unfortunately, as the size of the protein being studied increases, line broadening eventually makes it impossible to resolve individual peaks, necessitating alternative approaches for assigning peaks and obtaining distance constraints in larger molecules.

It is well known that spectra can be simplified by incorporating specific isotopically labeled amino acids into the protein. This can enable the resolution of otherwise indistinguishable peaks. Assignments can be made either by judicious application of hetero-nuclear multiple quantum coherence (HMQC) methods or by site directed mutagenesis. The HMQC approach detects the scalar coupling between the ^{13}C labeled carbonyl of one amino acid and the ^{15}N labeled amide of an adjacent residue. This approach was pioneered by Kainosho and Tsuji (1). Site directed mutagenesis has been used to assign resonance peaks in a number of proteins, beginning with the work of Jarema et al. on *lac* repressor (2). Simple and efficient protocols for site-directed mutagenesis have helped to make this approach viable for any cloned protein which can be expressed in culture. Most individual amino acid mutations have almost no effect on the overall structure of proteins (3). NMR spectra of mutant proteins provide an internal control for conservation of structure: any major change in structure will be accompanied by dramatic changes in the spectra.

Distance constraints can be obtained by measuring the effects of paramagnetic ligands, bound at specific sites in the molecule, on the NMR signals from previously assigned residues (4). This approach was pioneered by Dwek's group (5) and has since been applied to a number of relatively large proteins including Fab fragments (6) and bacterial D-lactate dehydrogenase (7).

We have used specific isotopic labeling and site-directed mutagenesis to assign resonance peaks for a number of residues in a human class mu glutathione transferase. This protein has a dimer molecular weight of ~50,000. We have obtained structural information by looking at the effects of a spin-labeled glutathione (a product analogue) on NMR line widths and have used this information to determine the approximate location of the hydrophobic substrate binding site in the enzyme.

RESULTS AND DISCUSSION

A human class mu glutathione transferase gene has been cloned and expressed in *E. coli* using a temperature sensitive expression vector. In this system protein expression is induced by increasing the temperature from 32 to 42°C. The bacteria are grown in a defined medium containing the appropriate labeled amino acids.

For assignment of tyrosine resonances the protein was expressed in an Aro^- host strain and the culture medium contained phenylalanine and tryptophan perdeuterated on the aromatic rings and d2,6-tyrosine. The aromatic regions of the resulting spectra (Fig. 1 *a*) contain signals from the three histidine rings and the 3,5-H tyrosine protons. Addition of spin-labeled glutathione (Fig. 1 *b*) broadens a number of the tyrosine lines. Site directed mutagenesis permits the identification of a number of unequivocal assignments on the basis of one dimensional spectra. Fig. 1 *c* shows the spectrum for the Y116F mutant which is clearly missing a peak at 6.3 ppm. Fig. 1 *d* is the spectrum of the mutant protein with spin label, the similarity to the spectrum of wild type protein (Fig. 1 *b*) reinforces our confidence that the mutation has had a negligible effect on the structure of the protein. Based on an analysis of the line broadening induced by spin labeled glutathione, tyrosine 116 is ~13 Å from the spin label at the hydrophobic substrate binding site. The distance constraints obtained in this manner are in agreement with the crystal structure obtained for a homologous class pi enzyme by Reinemer et al. (8). In those cases where the overlap between peaks is too great to allow assignment on the basis of one dimensional spectra, a total correlation spectroscopy

0006-3495/92/04/116/03 $2.00
Biophys. J. © Biophysical Society
Volume 62 Discussions 1992 116–118

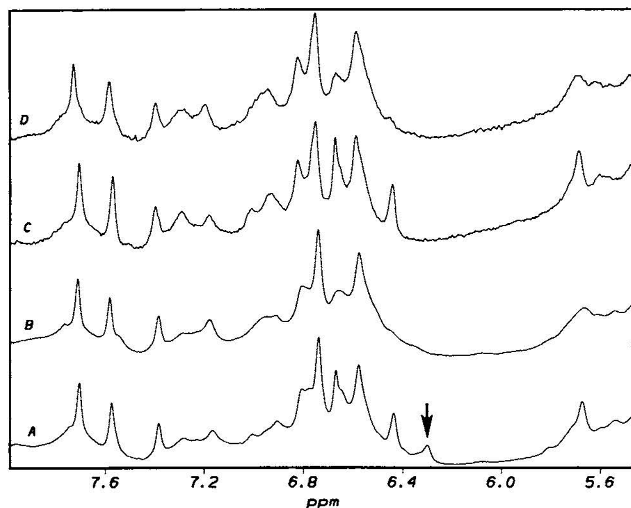

FIGURE 1 500 MHz proton NMR spectra of glutathione transferase. (*A*) Wild type protein, (*B*) wild type protein plus spin label, (*C*) mutant Y116F, and (*D*) mutant Y116F plus spin label.

(TOCSY) spectrum (of protein grown with nondeuterated tyrosine) has proven to be helpful.

Tryptophan residues can be observed using ^{19}F NMR. The bacteria are grown on media lacking aromatic amino acids. 5-^{19}F labeled tryptophan is added to the medium along with tyrosine, phenylalanine and *N*-phosphonomethyl glycine (an inhibitor of aromatic amino acid biosynthesis) upon induction. Fig. 2 *a* shows the ^{19}F spectrum of the wild type protein. Addition of spin label (Fig. 2 *b*) causes alterations in the chemical shift of the peaks around −47 and −53.5 ppm. Fig. 2 *c* of the W215Y mutant suggests that the peak at −53.5 ppm is that of W215. The reason for the effects of spin label on this spectrum (Fig. 2 *d*) are not clear at present. Although it is attractive to hypothesize that the peaks between −46 and −48 ppm arise from a single residue exchanging between different environments, it will be necessary to complete the set of mutant proteins and show that these peaks arise from a single residue before we can be confident of this explanation.

These observations indicate that it is possible to obtain structural information on a relatively large protein by combining NMR methods with site directed mutagenesis, specific isotopic labeling and the use of

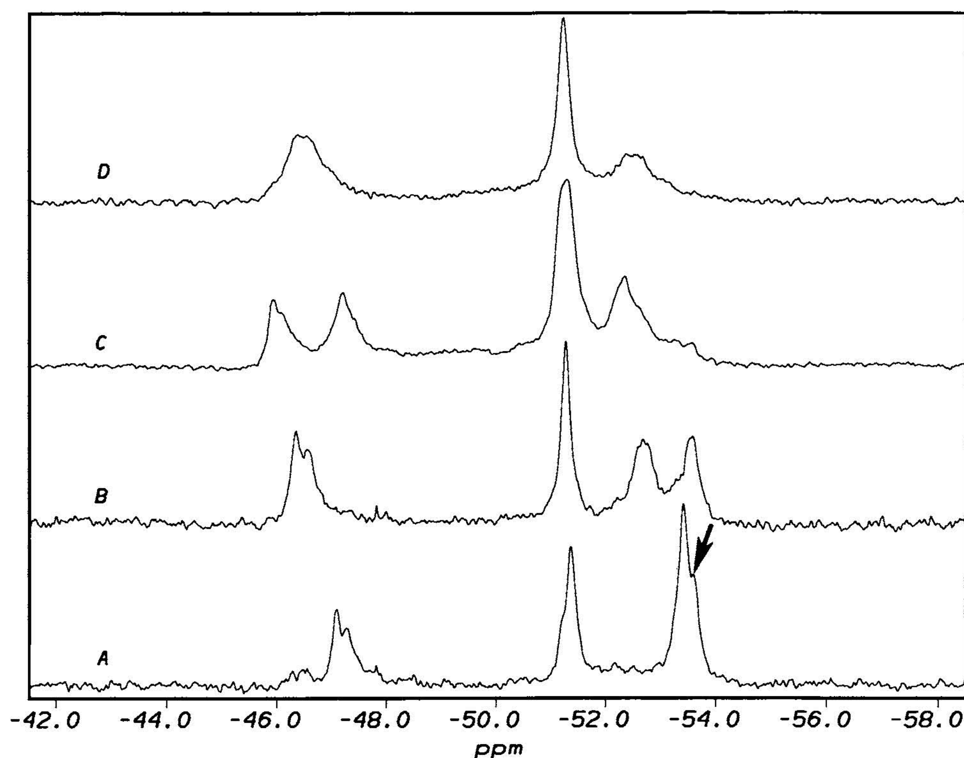

FIGURE 2 470 MHz fluorine NMR spectra of glutathione transferase. (*A*) Wild type protein, (*B*) wild type protein plus spin label, (*C*) mutant W215Y, and (*D*) mutant W215Y plus spin label.

paramagnetic broadening agents such as spin labeled substrates. Although, in this case, the experience with ^{19}F labeled tryptophan has been equivocal, earlier work by Peersen et al. (7) has demonstrated the utility of ^{19}F NMR for structural studies on proteins.

This work was supported by a research grant from the American Cancer Society (BC-703).

REFERENCES

1. Kainosho, M., and T. Tsuji. 1982. Assignment of the three methionyl carbon resonances in *Streptomyces* subtilisin inhibitor by a carbon-13 and nitrogen-15 double labeling technique. A new strategy for structural studies of proteins in solution. *Biochemistry.* 21:6273–6279.
2. Jarema, M. A. C., P. Lu, and J. H. Miller. 1981. Genetic assignment of resonances in the NMR spectrum of a protein: *lac* repressor. *Proc. Natl. Acad. Sci. USA.* 78:2707–2711.
3. Pakula, A. A., and R. T. Sauer. 1989. Genetic analysis of protein stability and function. *Annu. Rev. Genetics.* 23:289–310.
4. Kosen, P. A. 1989. Spin labeling of proteins. *Methods Enzymol.* 177:86–121.
5. Willan, K. J., D. Marsh, C. A. Sunderland, B. J. Sutton, S. Wain-Hobson, R. A. Dwek, and D. Givol. 1977. Comparison of the dimensions of the dinitrophenyl-binding immunoglobulin A myeloma proteins MOPC 315, MOPC 460 and XRPC 25 by spin-label mapping. *Biochem. J.* 165:199–206.
6. Anglister, J., T. Frey, and H. M. McConnell. 1984. Distances of tyrosine residues from a spin-label hapten in the combining site of a specific monoclonal antibody. *Biochemistry.* 23:5372–5375.
7. Peersen, O. B., E. A. Pratt, H-T. N. Truong, C. Ho, and G. S. Rule. 1990. Site-specific incorporation of 5-Fluorotryptophan as a probe of the structure and function of the membrane-bound D-lactate dehydrogenase of *Escherichia coli:* a ^{19}F nuclear magnetic resonance study. *Biochemistry.* 29:3256–3262.
8. Reinemer, P., H. W. Dirr, R. Ladenstein, J. Schaffer, O. Gallay, and R. Huber. 1991. The three dimensional structure of class pi glutathione S-transferase in complex with glutathione sulfonate at 2.3 Å resolution. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:1997–2005.