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Water has been shown to play an integral role in both structural and catalytic aspects of protein function. Strongly-bound water molecules are observed in high-resolution protein structures by x-ray diffraction studies of single crystals of protein molecules. The pilot studies described here were aimed at ascertaining the feasibility of detecting strongly-bound water molecules in solution using multidimensional heteronuclear NMR spectroscopy. 3D-(¹H, ¹⁵N)-HMQC-ROESY spectroscopy was implemented on a Bruker AM-500 spectrometer and spectra were collected on a series of related HPr protein molecules. Interactions involving both H₂O and -OH groups were reproducibly detected in the spectra. The results indicate that this approach is capable of yielding a limited amount of information concerning protein-water interactions and is not likely to become a generally-used technique.

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FINAL PROGRESS REPORT

Grant#: N00014-91-J-1766

R&T Code: 4412104

PRINCIPAL INVESTIGATOR: Prof. Rachel E. Klevit

INSTITUTION: University of Washington

GRANT TITLE: Study of Protein-H₂O Interactions by Multidimensional NMR Spectroscopy

REPORTING PERIOD: 4/1/91 - 3/31/93

AWARD PERIOD: 4/1/91 - 3/31/93 (Extended from 3/31/92)

OBJECTIVE: To detect and locate protein-bound water molecules within the established structure of the bacterial Histidine-containing protein (HPr) using three-dimensional nuclear magnetic resonance (3D-NMR) spectroscopy.

APPROACH: Using samples of natural isotope abundance and uniformly ¹⁵N-labelled HPr from *Bacillus subtilis*, we have attempted to observe direct ¹H-¹H nuclear Overhauser effect (NOE) interactions between solvent water and specific protons in the protein. The protein is dissolved in 90% H₂O /10% D₂O, resulting in an extremely strong solvent NMR signal. Since protein-bound H₂O molecules and bulk H₂O all resonate at the same frequency, methods for suppression of the water signal must be indirect, so that the water-protein NOE interaction to be observed is not simultaneously suppressed. As well, NOE crosspeaks between H₂O protons and protein protons will all lie along a single line in a two-dimensional NOESY spectrum. Three-dimensional NMR spectra obtained with ¹⁵N-labelled protein will spread the desired information out into a third (¹⁵N chemical shift) dimension, helping to overcome this spectral overlap problem. Distances obtained from NOE intensities can then be used to model the location of water molecules in the protein structure previously established by NMR.

ACCOMPLISHMENTS: In Year 01, our major efforts were on developing the three-dimensional NMR technology necessary to obtain the desired data on the instrumentation in our laboratory (a Bruker 2-channel AM-500 spectrometer). This proved challenging in that most of the reported 3D NMR experiments were performed on spectrometers with expanded capabilities. Nevertheless, we successfully worked to be able to perform these experiments on our spectrometer; and these experiments (e.g., 3D HMQC-NOESY, etc.) are now routinely performed on our instruments. This accomplishment has had a major impact on every project currently underway in my laboratory and was a direct benefit of Dr. Neil Jacobsen's efforts on the ONR project.

During the Extension period, the specific 3D experiment required to observe bound H₂O molecules, the 3D (¹H,¹⁵N)-HMQC-ROESY with (1-1) solvent suppression, was implemented and optimized. Dr. Jacobsen collected many such spectra to test for reproducibility and the effects of parameters such as sample temperature and pH. Once optimal conditions were found, 3D data sets were collected on a number of different but related HPr proteins for analysis.

Analysis of the data sets required the development of a series of software tools. Dr. Jacobsen wrote, tested, and documented a package of macros and awk programs designed

to take 3D NMR data from its unprocessed, collected form to a number of different formats useful for analysis. This package is now used by all members of my research group. He also developed tools for measuring 3D peak volumes which are particularly useful for both the H₂O project and for all projects in which information from multidimensional NMR spectra must be extracted.

The essence of the approach pursued in this pilot project is that H₂O molecules that are bound to protein molecules with lifetimes in the millisecond range will cross relax (i.e., will exhibit NOE peaks) to protons in the protein. Therefore, all the desired information is located on one plane of the 3D-HMQC-ROESY spectrum, at the resonance frequency of H₂O. In our Progress Report last year we showed the H₂O plane for the phosphotransfer protein, *E. coli* HPr, that is being studied in detail by NMR in my research group. There are many reproducible cross peaks in the plane of significant intensity. However, in addition to H₂O protons, almost all sidechain -OH groups (i.e., from Ser, Thr, and Tyr) also resonate at this frequency. Thus, many of the cross peaks that appear in the figure may arise from protein -OH groups, rather than bound solvent molecules. Figure 1 shows the data as a histogram of peak intensity (in arbitrary units) in the H₂O/-OH plane along the *E. coli* HPr protein sequence, with all Ser, Thr, and Tyr residues circled. It is clear from this representation that there are -OH groups throughout the primary structure of the protein that give strong signals in this experiment. If bound H₂O are to be unambiguously identified, it will be necessary to distinguish them from the -OH groups. This can, in principle, be accomplished because the pH-dependence of -OH exchange is well-known. Wüthrich and co-workers have demonstrated that collection of a series of 3D-HMQC-ROESY spectra as a function of sample pH allows the cross peaks arising from -OH groups to be distinguished. This is, however, an extremely instrumentation-intensive effort (each 3D spectrum takes ~3.5 days of spectrometer time to collect), and so is not likely to become a common practice in most NMR groups where spectrometer time is the limiting parameter.

A careful examination and comparison of our own data on HPr has revealed several intriguing cross peaks to residues that are not close to any -OH groups in the 3D structure of the protein. The same cross peaks, involving residues Gln 3, Lys 72, and the sidechain of Gln 4 are observed reproducibly in both wild-type HPr and a single point mutant. This increases our confidence in the observation and we intend to pursue this point in the future as we continue to study and refine our understanding of HPr.

In summary, the technique implemented and applied to the well-studied protein system, HPr, in this project is capable of yielding information concerning strong interactions between the protein and H₂O molecules. However, as mentioned above, due to the unfortunate redundancy of the information with interactions involving hydroxyl groups, unambiguous information requires the collection of many 3D spectra, each taking about 3.5-4 days of spectrometer time. For most NMR groups, where spectrometer time is the limiting commodity, it will probably be deemed that the amount of new information resulting from these experiments does not justify the amount of spectrometer time necessary, except in cases where a specific question involving solvent molecules is involved. However, in cases where instrument time is plentiful, the technique is likely to be embraced and applied. Indeed, this has already been shown to be the case in several major NMR labs, including those of Prof. Kurt Wüthrich (ETH, Zurich), Dr. Steven Fesik (Abbott Labs), and Dr. Peter Wright (Scripps Institute).

SIGNIFICANCE: Due to their relatively rapid exchange with bulk solvent protons, both bound H₂O protons and sidechain hydroxyl protons resonate at the same position as bulk H₂O. Therefore, an NOE (or ROE) interaction observed between a protein proton and

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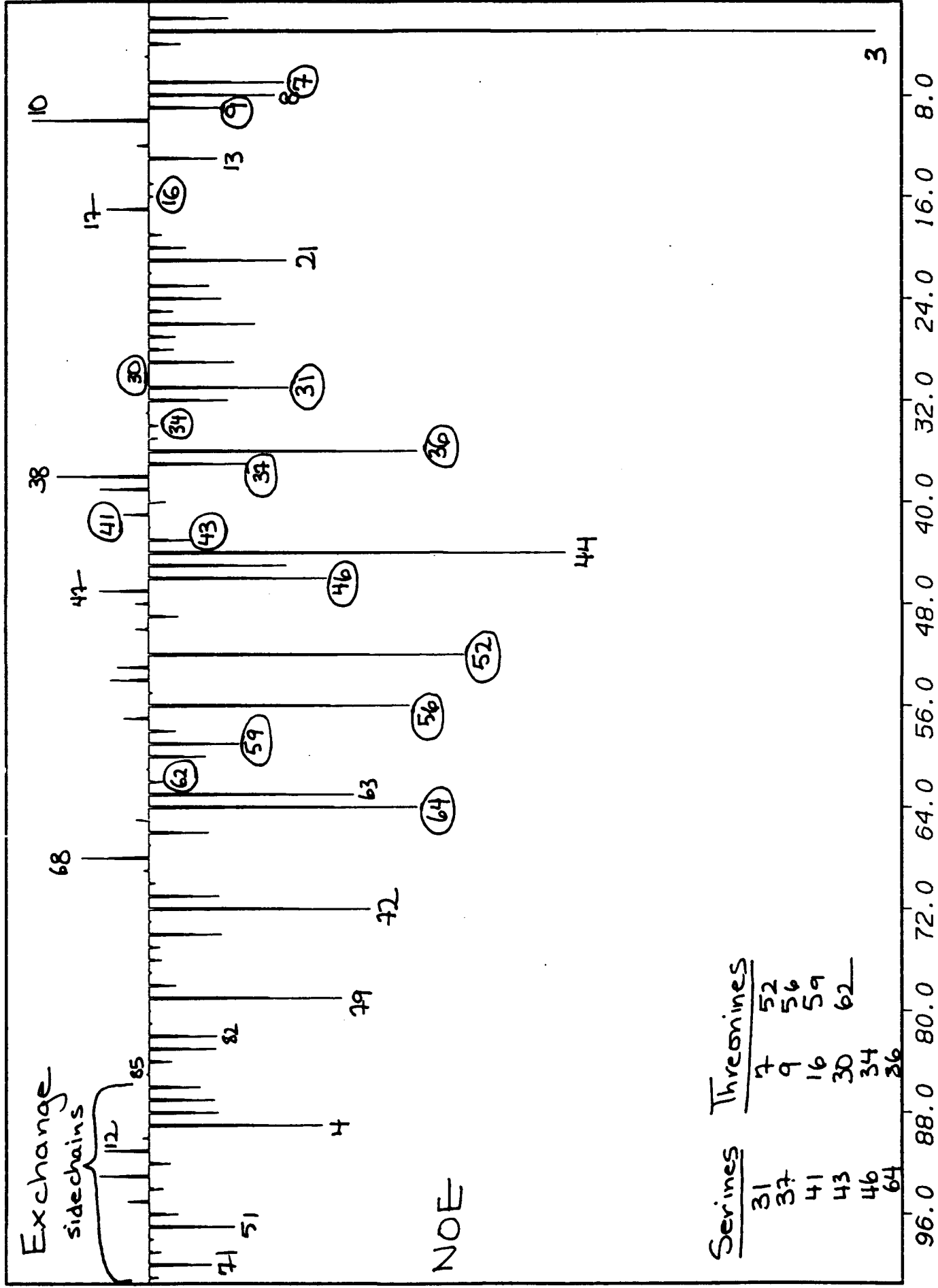
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something that resonates at the solvent signal frequency could arise from either a bound H₂O or from an -OH. The NOEs actually observed must be interpreted in the context of a refined three-dimensional protein structure. HPr has 16 hydroxylic residues distributed fairly evenly over the 88 residues of the protein, so that it is crucial to have a very well-defined structure in order to interpret the NOEs observed. The 3D experiments obtained in the process of this work have already been useful in our efforts to refine our current HPr structure. This solution structure reveals that there are indeed regions of the protein, notably in its interior, where no -OH groups reside. Therefore, we believe that it is possible to observe a strongly-bound H₂O molecule using these approaches, if one (or more) exists.

PUBLICATIONS. ABSTRACTS. PATENTS:

None directly stemming from this ONR pilot project. However, I expect to incorporate the results obtained from these studies in a paper describing the refined structure of HPr in the coming year. ONR Support will be acknowledged in this paper.



ANNUAL REPORT QUESTIONNAIRE
(for ONR use only)

Principal Investigator Name: Rachel E. Klevit

Institution: Univ. of Washington

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Number of ONR supported

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Papers or reports in non-refereed publications: 0

Books or book chapters published: 0

Number of ONR supported patents/inventions

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Granted: _____ Patent name and number: _____

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AWARDS/HONORS TO PI AND/OR TO MEMBERS OF PI'S RESEARCH GROUP (please describe):

Van't Hoff Award (Royal Netherlands Academy of Arts and Sciences) 1991

Equipment purchased on grant (number and description of items costing >\$1,500):

2 Silicon Graphics Indigo Workstations

STUDY OF PROTEIN-H₂O INTERACTIONS

BY MULTIDIMENSIONAL NMR SPECTROSCOPY

Objective:

- use NMR spectroscopy to detect interactions between specific nuclei in a protein and H₂O molecules
- highly-ordered H₂O molecules are found in crystalline proteins
- Is ordered H₂O an integral part of protein solution structures?
- What aspects of protein structure dictate the interaction?
- How much stability do such interactions afford?

Accomplishments:

- 3D NMR spectra of HPr → refinement of solution structure
- NOEs observed between protein protons and either -OH or H₂O

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