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FOLLOWING MACROMOLECULAR INTERACTIONS AND SUGAR METABOLISM USING SITE SPECIFIC ^3H LABELLING AND NMR SPECTROSCOPY

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SUMMARY

In this paper we discuss the application of ^3H NMR to biological problems. Two specific examples will be described; first, analysis of the binding of maltose to its transport protein from *E. coli*, called MBP; and second, following the glycolytic metabolism of glucose in erythrocytes. In both of these cases the unique properties of ^3H for magnetic resonance make possible observations which are difficult with other methods.

Maltose binding protein, MBP, is involved in transport of maltodextrans (maltose and its oligomers) across the periplasmic space in *E. coli*. It interacts with the maltose specific pore, Lam B, to facilitate passage of the maltose into the interior of the cell, and also with a chemotactic receptor. MBP is a soluble protein of 40kD, with known primary sequence, which has an association constant for maltose of about 10^6, increasing somewhat for the longer maltose oligomers[1]. We were interested in characterizing the general binding mode of maltose to MBP, and also in determining the kinetic parameters for the association - dissociation reaction. The initial step involved acquiring a series of spectra while titrating maltose into a sample of the protein. The association of maltose with MBP causes significant change in chemical shifts for resonances throughout the spectrum. Among the affected resonances is a methyl group with a chemical shift of ca. -1 ppm. By titrating a sample to have 1/2 mole of maltose per mole of protein, and following the resulting pair of lines with increasing temperature we were able observe linebroadening associated with chemical exchange (transfer of maltose from one MBP to another), and could fit the curves for temperatures of 30-60°C to give an "off rate". However for lower temperatures and longer maltose oligomers the rates were too low to give a significant effect on the spectrum. In addition, since there are protein lines everywhere in the spectrum which shift upon addition of the sugar, it was impossible to identify lines from the bound maltose, even using difference spectra, Figure 1A,B.

To solve these problems we took advantage of the relative ease of labelling sugars at the reducing end, and the high sensitivity of ^3H NMR. A sample of about 43 mg of maltose (0.12 mmoles) was dissolved in water and reacted with carrier free tritium gas over a $Pd/BaSO_4$ catalyst for 2 hours at ambient temperature[2]. The excess tritium gas was removed by evacuation, the sample flushed with nitrogen, the resulting product was washed with methanol, then lyophilized

twice from water to remove any residual labile tritium. The labelled maltose was then analysed by HPLC on a Waters CHO column, with both radioactivity and refractive index detection. The major peak obtained eluted with the same retention time as the starting material, there was a second, minor, nontritiated peak which eluted slightly earlier. From the specific activity of the product (4.7 Ci/mmole) we deduce that labelling occurs to approximately 16%, due to inefficiency of the exchange reaction. This is relevant only in that it decreases the number of spins present in the NMR experiment.

The proton decoupled ^3H spectrum of the labelled maltose shows two lines, Figure 1C, from the α and β anomers at 5.2 and 4.6 ppm (from DSS) respectively, showing the normal ratio of 40:60. These spectra were acquired at a ^3H frequency of 320 MHz on an IBM Instruments AF-300 spectrometer, 5 mm sample tube, from a sample which contained a total of 800 μCi of tritium. When MBP (ca. 1 mM solution, in phosphate buffer at pH 7.0) is titrated with labelled maltose two lines again appear, but now at 3.5 and 3.4 ppm, Figure 1D. The lines are broadened relative to free maltose since the sugar now tumbles with the longer correlation time of the protein. When further additions are made exceeding a 1:1 mole ratio maltose to MBP, free maltose is observed at the same chemical shifts as in the absence of protein, Figure 1E. The dramatic upfield shifts of the resonances can only be explained by proximity of an aromatic group on the protein - from the size of the shift probably tryptophan. The degree of shift of the two anomers is slightly different, which could reflect either a difference in the position at which the sugar binds, or just the differences in position of the triton on the sugar, or a combination of the two. It is interesting to note that there is very little anomeric specificity in the binding of the sugar, during the titration the resonance intensities of the bound forms of the two anomers remain at their equilibrium value (spectra are acquired over a time of about 8 hrs, long compared with the time constant for equilibration of the anomers, ca. 2.5 hrs). The large separation in chemical shift of bound and free maltose makes possible several other experiments as well. It is possible to saturate one of the resonances of the maltose and then look for effects on the bound form, a saturation transfer experiment. When the rate constant is comparable to the inverse of the spin lattice relaxation time, T_1, then the bound form resonance will also become partially saturated. From the degree of saturation, and an independent determination of the T_1 value, the rate constant can be determined. Since the separation between the free resonances of the α and β anomers is fairly large this can be done for the two independently. The rates for transfer between the free and bound of each anomer are very similar. The saturation transfer experiment works well even for rate constants a factor of 100 less than gives rise to sufficient linebroadening to be observed, making it applicable to the longer maltose oligomers.

We have also carried out heteronuclear NOE experiments, saturating the tritium resonances of the maltose, and looking for changes in intensity of proton resonances of MBP. Such effects will

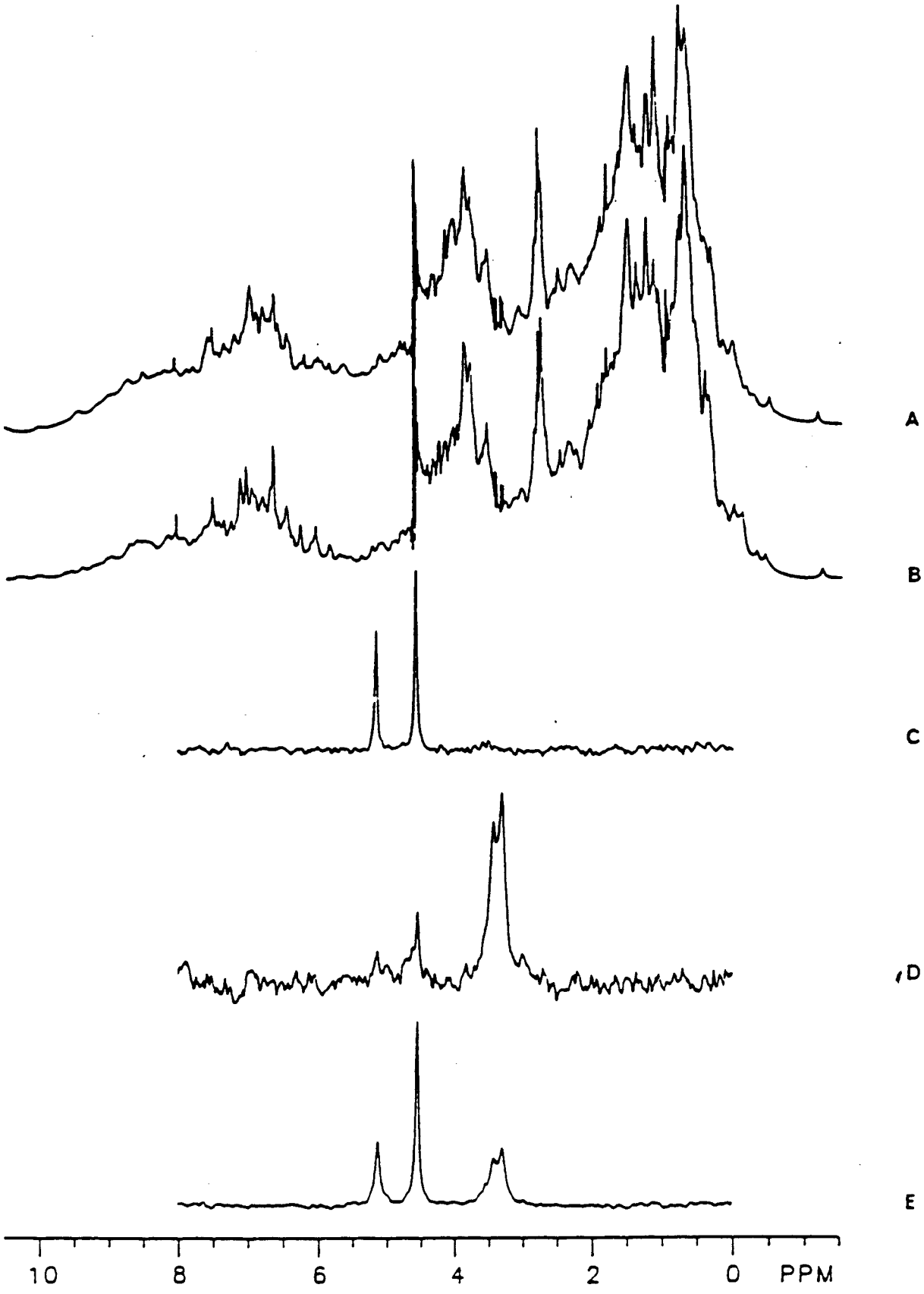

Figure 1. A) ^{1}H NMR spectrum of MBP with 1 mole equivalent of maltose added, B) MBP with no maltose added, C) ^{3}H NMR spectrum of selectively labelled malose, D) labelled maltose in the presence of excess MBP, E) labelled maltose in excess relative to MBP.

be seen when the distance between the saturated triton, and the neighboring protons are $\leq$ 4Å. When NOEs are seen they can be qualitatively evaluated based upon the chemical shift of the proton, however this does not provide an assignment to a specific residue on the protein. This further step could be accomplished with simultaneous use of other isotopic labels on the protein, and/or site directed mutagenesis.

Another set of experiments were done with tritium labelled sugar, in this case aimed at probing metabolism in erythrocytes. In this case glucose was labelled at the C1 position in a manner analogous to maltose. The spectrum of the free sugar in water solution again shows similar separate resonances for the α and β anomers, with intensity ratio 40:60, in good agreement with values measured previously with other methods. A sample of this glucose was incubated with a suspension of packed rat erythrocytes (pH 8.0 phosphate buffer, isotonic glucose, with a small amount of deuterium oxide added for locking) at 37°C. Under these conditions the cells are essentially anaerobic, and metabolize the glucose using the glycolytic pathway[3]. As a function of time the lines from glucose are reduced in intensity, and three new lines grow in, Figure 2. One of these increases in intensity early in the reaction, but then decreases again at later times, the expected behaviour of an intermediate. The other two lines grow in, reaching stable plateau values at long times. From their chemical shifts these two products can clearly be identified as lactate and water. Lactate, containing the tritium at the methyl position, is the expected major product of glycolosis. The chemical shift of the intermediate is unfortunately ambiguous, lying close to both 2,3-diphosphoglycerate and fructose-1,6-diphosphate, both representing intermediates on this pathway which can be expected to be present in significant concentrations. The appearance of tritium in the water is unexpected from the normal glycolytic pathway, in which the proton from the C1 position of glucose is retained all the way to lactate, the final end product. However some of the sugar may go through an alternate pathway after conversion to glucose-6-phosphate. This occurs through dehydration of the sugar to 6-phosphoglucono-δ-lactone, releasing the triton from the C1 position, which then enters the hexose monophosphate shunt. By comparing the resonance intensities of these products with time, rate constants can be determined for the various steps, including those through the shunt, Figure 2.

There are several important facts to be noted in this study. First, the sensitivity of ^3H for NMR purposes is significantly higher than other available isotopes[4] (e.g. ^{13}C), allowing the studies with higher time resolution, and at lower concentration. Second, there is no natural background for tritium, all of the signals arise directly from the labelled reactants which are fed into the system, at known times and concentrations, and from the reaction products of these. It is possible to see the production of even very small amounts of a product like HOT, where the tritium signal represents a concentration of about 1 mM, detected in the presence of 110 M water. The alternate isotope for studies of hydrogen, deuterium, suffers from the NMR view point from low

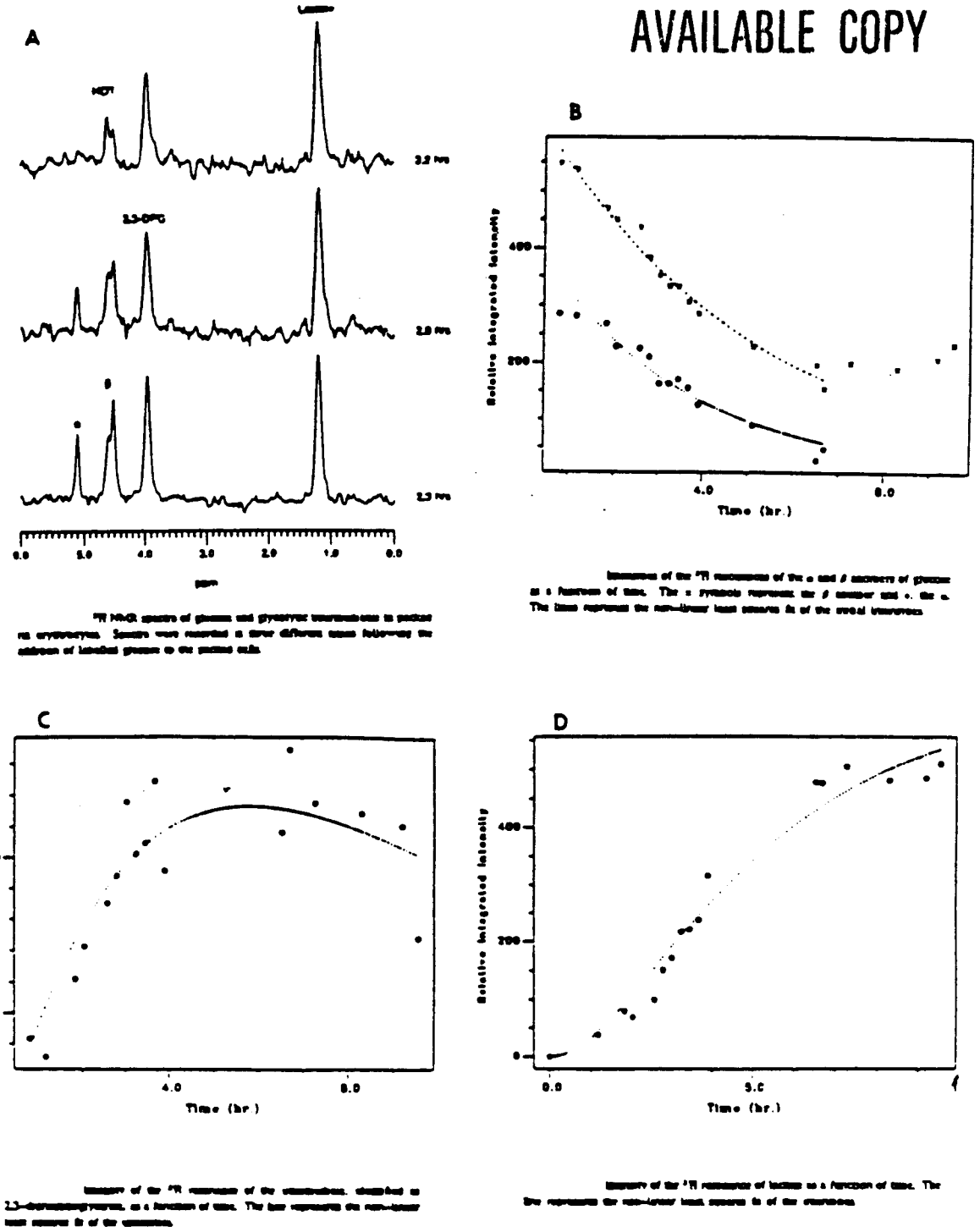

Figure 2. A) ^{3}H NMR spectra of tritiated glucose which has been incubated with packed erythrocytes for the times indicated. Peaks are labelled with the compound from which they originate. B) Graph of the intensities of the α and β glucose resonances as a function of time of incubation with erythrocytes. C) Graph of the intensity of the major intermediate, as in B. D) Graph of the intensity of the lactate product as in B).

sensitivity and resolution. This is especially true for any study which involves a macromolecule, the slow tumbling making deuterium lines extremely broad. In addition since the dominant relaxation mechanism for deuterium is quadrupolar, NOE determinations are impossible, and sensitivity in saturation transfer is reduced due to rapid spin-lattice relaxation. Although there are problems with handling radioactive samples, in many cases the ease of labelling, and the utility of background-free spectra for examining binding and metabolic phenomona make the effort required worthwhile.

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