THE USE OF SEMISYNTHETICALLY-INTRODUCED ^{13}C PROBES FOR NUCLEAR MAGNETIC RESONANCE STUDIES ON INSULIN

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1. Introduction

This paper is intended to demonstrate the feasibility of employing protein semisynthesis – the chemical synthesis of a modified protein from suitably-protected fragments derived by proteolysis (e.g. [1, 2]) – in nuclear magnetic resonance studies. We have chosen to investigate the NMR of ^{13}C [3–5] recognising, as have other workers [6] the advantages that would derive from isotopic enrichment at a specific site if it were possible to avoid using some bulky reagent to introduce the enriched group. We report the removal of phenylalanine (B-1) from insulin and its replacement with glycine enriched in ^{13}C approx. 90-fold over the natural (1%) of the isotope. The result is promising in that the complex natural-abundance spectrum of insulin is simplified to a single peak. The chemical shift of this peak varies significantly with pH.

2. Methods

2.1. *The insulin derivative*

The method of Borras and Offord [2] was used to prepare porcine insulin that lacked the amino-terminal phenylalanine of the B-chain and that was reversibly protected by trifluoracetylation of the other two amino groups (the α-amino group of residue A-1 and the ϵ-amino group of residue B-29)*. Thus the only free amino group was that of the valine (residue B-2) to which the phenylalanine had been adjacent. [1-^{13}C]Glycine (60% isotopic abundance) and [2-^{13}C]glycine (86% isotopic abundance) were obtained from Prochem Ltd., Deer Park Road, London, S.W. 19, England, and coupled to the derivative to produce a new B-1 residue as follows.

2.2. *Amino protection of the glycine*

18.8 mg of either of the ^{13}C-labelled glycines was trifluoracetylated on the α-amino group by the method of Weygand and Geiger [7]. (This method is particularly suitable for small quantities of material.) It involves reaction between the amino acid and trifluoracetic anhydride in anhydrous trifluoroacetic acid. The final purification of the derivative is by sublimation. The yield of trifluoracetyl[1-^{13}C]glycine (m.p. 120°) was 90% and of the 2-^{13}C material (m.p. 119°) was 62%. The recorded m.p. of the material without isotopic enrichment [8] is 120–121°.

2.3. *Activation of the trifluoracetyl glycine to the hydroxysuccinimide esters and coupling to the insulin derivative*

This was carried out as previously described [2] for the coupling of the natural and [^{3}H]phenylalanine except that a 7.6-fold molar excess of the activated amino-acid derivative was used (the concentration of this derivative in the reaction mixture was 0.25 mM, that of the insulin derivative was 1.9 mM) and coupling was carried out at room temperature overnight. Small-scale experiments show that reaction was substantially complete after 10 min. The removal of the trifluoracetyl groups by 1 M ammonia was carried out

* The sequential information and the numbering system for the residues is taken from [9]. The insulin was the gift of Dr. Schlichtkrull, Novo Terapeutisk Laboratorium, Copenhagen and came from batch S 311069.

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Table 1
Glycine-to-phenylalanine ratios of the insulin and its derivatives.

Sample	Gly:Phe ratio		Dansyl amino acids
	Observed*	Expected	
1. Insulin	4.0:3.3	4:3	Gly, Phe, (ε)Lys, *O*-Tyr
2. Trifluoracetyl *des*-Phe derivative	4.0:2.3	4:2	Val, *O*-Tyr
3. [1-^{13}C]Gly-(B1)-insulin	5.0:2.3	5:2	Gly, *O*-Tyr, εLys
4. [2-^{13}C]Gly-(B1)-insulin	5.0:2.2	5:2	Gly, *O*-Tyr, εLys

* Values for phenylalanine that are 10% high are characteristic of all our analyses of insulin and its derivatives.

as described by the same authors as was the characterisation of the products by isoelectric focussing, amino acid analysis and dansylation (see table 1). The final products were freed from any uncombined glycine by dialysis and precipitation at pH 5.5 after the treatment with ammonia.

2.4. *Nuclear magnetic resonance spectroscopy*

The samples of [^{13}C]insulin were dissolved in water (20% in D_2O to provide an internal field frequency lock) at a concentration of 3.3 mM in the case of the [1-^{13}C]Gly derivative and 2–4 mM in the case of the [2-^{13}C]Gly derivative. Natural insulin was treated in the same way, but dissolved at a concentration of 6 mM. Sample volumes were 2.3 ml. It was found to be necessary to remove any traces of carbon-containing, low molecular-weight materials, such as the acetone and citrate buffer used in the crystallisation procedure of Schlichtkrull [10] to which the samples had been exposed. Such compounds, if present in any quantity, give very sharp resonances which may be comparable in visibility to the protein peaks.

The samples were placed in a Brüker NMR spectrometer operating at 22.63 MHZ. This instrument was provided for the Oxford Enzyme Group by the Science Research Council. (It was set up and operated by Dr. I. Campbell.) The Fourier-transform mode [11, 12] was employed with computer accumulation and broad-band proton decoupling. Typical runs involved the accumulation of 25,000 scans, which took 3 hr. The sample temperature was about 30°.

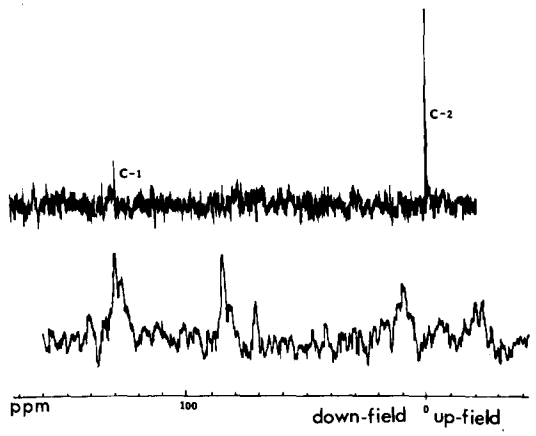

Fig. 1. Tracing of natural-abundance NMR spectra of 6 mM insulin (lower trace) and 500 mM glycine (upper trace). The chemical shifts in all figures are normalised to the position of the resonance of C-2 of glycine at pH 7 in 20% D_2O.

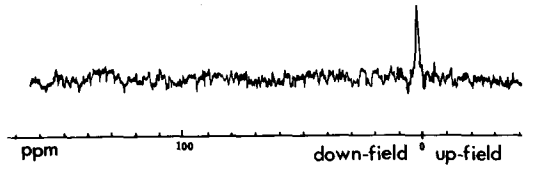

Fig. 2. Tracing of NMR spectrum of semi-synthetic [2-^{13}C]-Gly-(B-1)-insulin (2 mM). For other details see text.

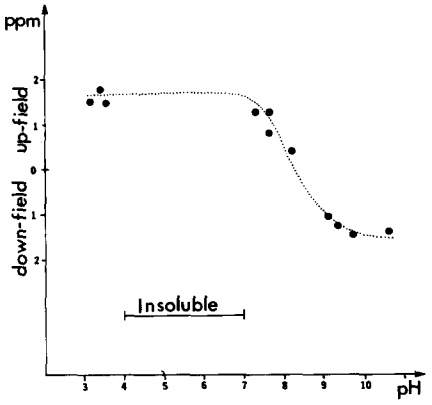

Fig. 3. The variation with pH of the peak position of the resonances obtained as in fig. 2. The region of extreme insolubility of insulin is indicated.

3. Results and discussion

Fig. 1 shows a spectrum of the natural-abundance ^{13}C in ordinary insulin and, superimposed, the natural-abundance ^{13}C of glycine (500 mM). The assignments of the resonances given in this figure are based on tabulated [3] values which were derived from the spectra of the free amino acids. We do not observe for insulin the same marked departure from the prediction of the ratio of the intensities of the carbonyl and aromatic regions to those of the other resonances that was observed for hemoglobin [5].

The disparity in the heights of the two glycine peaks in fig. 1 is a consequence of the Overhauser enhancement [12] of the 2-C resonance by the protons attached to that atom and perhaps also different times of relaxation between the nuclei. Studies on 1-^{13}C material are therefore at a disadvantage. In addition, all the resonances of the 50 or so carbon atoms in the carbonyl groups of the various residues in the two chains are very close together. These two disadvantages were reflected in the uninformative results that were obtained with the 1-^{13}C material.

Fig. 2, however, shows a spectrum of the semi-synthetic [2-^{13}C]Gly-(B1)-insulin and fig. 3 shows the chemical shift of the peak as a function of pH. In fig. 2 the peak width at the half height is 16 Hz, a much larger figure than that observed for the free amino acid in fig. 1. Approximate calculations show that much of this additional broadening could be accounted for simply by the slower rate of tumbling of the macromolecule. The insulin will be in the form of a hexameric (M.W. 36,000) or higher state of aggregation under the conditions of the experiment.

We do not wish to comment, on the basis of these initial experiments, on the value for the pKa of the chemical shift that can be derived from fig. 3 (approx. 8.2) except to say that the rather high value that is observed is not incompatible with the position of residue B-1 in the hexamer. This is because it is in close contact with a negatively-charged side chain on another adjacent monomer, and the resulting interaction would be expected to raise the pKa.

We conclude that the semisynthetic method has some value in NMR studies and propose to investigate the response of the resonance at residue B-1 and other positions to various changes in conditions. (These could usefully include the replacement of the zinc ions that stabilise the hexamer with paramagnetic ions.) The present studies were carried out with [^{13}C]glycine, because this was the only material commercially available to us. Now that the feasibility of the approach has been demonstrated it would clearly be much more satisfactory to synthesize L-phenylalanine (enriched in the α carbon or side chain) for use at position B1.

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References

[1] R.E. Offord, Nature 221 (1969) 37.
[2] F. Borras and R.E. Offord, Nature 227 (1970) 716.
[3] W. Horsley, H. Sternlicht and J.S. Cohen, J. Amer. Chem. Soc. 92 (1970) 680.
[4] F.R.N. Gurd, P.J. Lawson, D.W. Cochran and E. Wenkert J. Biol. Chem. 246 (1971) 3725.
[5] F. Conti and M. Paci, FEBS Letters 17 (1971) 149.
[6] M.H. Freedman, J.S. Cohen and I.M. Chaiken, Biochem. Biophys. Res. Commun. 42 (1971) 1148.
[7] F. Weygand and R. Geiger, Chem. Ber. 92 (1956) 295.
[8] F. Weygand and E. Leising, Chem. Ber. 87 (1954) 248.
[9] H. Brown, F. Sanger and R. Kitai, Biochem. J. 60 (1955) 556.
[10] Schlichtkrull (1958), Thesis, University of Copenhagen.
[11] R.R. Ernst and W.A. Anderson, Rev. Sci. Instrum. 37 (1966) 93.
[12] E.G. Paul and D.M. Grant, J. Amer. Chem. Soc. 86 (1964) 2977.