¹H n.m.r. studies of insulin

Assignment of resonances and properties of tyrosine residues

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The assignment of the aromatic ¹H n.m.r. resonances of the four tyrosine residues of bovine 2-zinc insulin is reported, based on double resonance techniques, use of Hahn spin echo pulse sequences and examination of specific derivatives nitrated at tyrosines A14 and A19 as well as des-(B26–B30)-insulin. Titration curves of the four tyrosine residues show that residues A14 and B16 have normal pK' values of 10.3–10.6 in solution, consistent with their accessibility to solvent in monomer and dimer in the crystal. Tyrosine residues A19 and B26 have pK' values of 11.4 and exhibit other features in their titration curves that are consistent with limited accessibility to solvent and a nonpolar environment. The *meta* protons of residues B16 and B26 both observe the titration of a nearby tyrosine residue, probably A19. Interpretation of the n.m.r. data obtained in solution is consistent with the crystallographic data for the monomer and dimer obtained on insulin crystals [Blundell, Dodson, Hodgkin & Mercola (1972) *Adv. Protein Chem.* **26**, 279–402].

The four tyrosine residues of insulin have been shown to play an important role in the organization of 2-zinc insulin hexamer, both in the crystal (Blundell *et al.*, 1972) and in solution (Strickland & Mercola, 1976; Mercola & Wollmer, 1981). Three of them, A14, B16 and B26, are involved in contacts between insulin molecules during association to form the hexamer, but tyrosine A19 does not participate in the aggregation process. Similarly, tyrosines A14 and A19 play an important structural role in the transformation of 2-zinc insulin to 4-zinc insulin (Bentley *et al.*, 1976). Detailed studies using c.d. have been made in solution (Strickland & Mercola, 1976; Mercola & Wollmer, 1981; Wollmer *et al.*, 1977) and there has been one very recent ¹H n.m.r. study using the photochemically induced dynamic nuclear polarization method (Muszkat *et al.*, 1984) on the tyrosine residues. Other n.m.r. studies of native insulin (Kowalsky, 1962; Bradbury & Wilairat, 1967; Bradbury & Brown, 1977; Williamson &

Williams, 1979; Bradbury *et al.*, 1981; Cheshnovsky *et al.*, 1983) have been made difficult by the insolubility of the hormone between about pH 4 and 7 and its complex aggregation behaviour above pH 7 (Blundell *et al.*, 1972) which will be discussed elsewhere (J. H. Bradbury & V. Ramesh, unpublished work). In the present paper we report the complete assignment of the aromatic ¹H n.m.r. resonances of the tyrosine residues of 2-zinc insulin by means of selective nitration of tyrosine and also by use of a derivative in which tyrosine B26 is deleted. This has allowed determination of pK' values of the tyrosine residues and comparison of their accessibilities in solution with that observed in the crystal.

Experimental

Materials

Insulin (Sigma, bovine pancreas, crystallized) typically contained 0.42–0.44% Zn, in excess of the stoichiometric amount (0.35% Zn) for 2-zinc insulin hexamer (Schlichkrull, 1958). The zinc was removed by extensive dialysis at pH 3.0–3.5 and 20°C (Cunningham *et al.*, 1955) and the correct amount of zinc (0.35%) was added to give 2-zinc insulin hexamer. The sample was lyophilized and the zinc content was checked routinely by atomic

Abbreviations used: C³.A14-nitroinsulin, C³.A14, C³.A19-bis(nitro)insulin, insulin with 3-nitrotyrosine residues at A14, and A14 and A19, respectively.

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absorption spectroscopy. A sample of des-(B26-B30)-insulin was obtained from Dr. G. Dodson. Samples of $^2\text{H}_2\text{O}$ (>99.8% and 100.0%) were obtained from the Australian Atomic Energy Commission and Merck, Sharp and Dohme (Canada) respectively. Samples of ^2HCl (Aldrich), NaO^2H (Merck, Sharp and Dohme), tetranitromethane (Koch-Light) and all other reagents used, were of analytical grade purity.

Methods

Spectra were obtained with a Bruker HX-270 n.m.r. spectrometer with an Oxford Instrument Co. superconducting magnet using 4096 data points, pulse length $8\text{ }\mu\text{s}$, acquisition time 0.5 s, and gated decoupling to reduce the size of the H^2HO resonance. In order to sharpen the spectra, the free induction decay was usually manipulated by a

Fig. 1. ^1H n.m.r. spectra at 270 MHz of the aromatic region of 2-Zn insulin (3.47 mM in $^2\text{H}_2\text{O}$) at 37°C as a function of pH

weighting function using the sine-bell multiplication (De Marco & Wuthrich, 1976) or the trapezoidal multiplication correction. The Hahn spin echo pulse sequence ($90^\circ-\tau-180^\circ-\tau$), with $\tau = 60$ ms, was used to invert the tyrosine doublets ($J = 8$ Hz) and hence to simplify the overlapping aromatic ¹H n.m.r. resonances (Campbell *et al.*, 1975a). Insulin solutions (1–2%) in ²H₂O at 37°C were examined and chemical shifts were reported using *p*-dioxan as an internal reference with δ 3.761. A Beckman model 4500 pH meter with an Ingold combination electrode was used to measure pH before and after each n.m.r. measurement and the results normally agreed within 0.05 pH unit. Results are pH meter readings uncorrected for deuterium isotope effects (Glasoe & Long, 1960). The pK' values of tyrosine residues were determined by using a computer program (Carver & Bradbury, 1984).

The method of Morris *et al.* (1970) using tetranitromethane was followed to prepare mono-3-nitrotyrosine insulin and bis-3-nitrotyrosine insulin. Yields were about 15% for each derivative and amino acid analyses (Ramesh, 1982) confirmed the degree of nitration of the tyrosine residues. Morris *et al.* (1970) showed that the mononitrated derivative was substituted at tyrosine A14 and the dinitrated derivative at tyrosines A14 and A19.

Results

¹H n.m.r. spectra of the aromatic region of insulin at high pH previously obtained (Bradbury *et al.*, 1981) were considerably sharpened by using the sine-bell multiplication and results are shown

in Fig. 1. At pH values above 10.8 spectra were collected rapidly to avoid denaturation of insulin. For example, at pH 11.5 it was found that no appreciable amount of denaturation occurred within 30 min after preparation of the sample. The effects of high pH on insulin were shown to be reversible by lowering the pH back to 9.85, when the original n.m.r. spectrum was observed. Prolonged exposure to high pH (2 h at pH 11.5 or 4.5 h at pH 10.8 at 37°C) caused denaturation and gave readily observable changes in the n.m.r. spectrum, including the occurrence of a single, denatured H-2 histidine resonance (Ramesh, 1982) between the two downfield B5 and B10 resonances in Fig. 1.

All our measurements were made on samples which did not show this denatured histidine resonance. It was also noted that the spectra of both 2-Zn insulin and Zn-free insulin were essentially the same above pH 10.3, due to the dissociation of 2-Zn insulin hexamer to produce dimeric insulin which no longer binds zinc (Blundell *et al.*, 1972). Above pH 11 in Fig. 1, there is the progressive appearance of resolved doublets due to tyrosine residues and the coupling together of these doublets was achieved by spin decoupling.

The respective *ortho* (3,5) and *meta* (2,6) proton doublets of the four tyrosine residues were linked by double-resonance experiments shown elsewhere (Ramesh, 1982). In Table 1 the pairs of doublets identified by spin decoupling experiments are given as well as the data from pH titration and assignment (see below). Further elucidation of the spectrum was obtained by a Hahn spin echo experiment (Ramesh, 1982) in which the tyrosine doublet resonances were inverted, but resonances E, E' (6.85, 6.87 p.p.m.; see Fig. 1) were not

Table 1. ¹H n.m.r. titration data for the tyrosine residues of 2-zinc insulin at 37°C

Assignment	Numbering of resonances (Figs. 2–4)	N.m.r. parameters†			
		pK'	δ_A (p.p.m.)	δ_B (p.p.m.)	J (Hz)
A14 <i>ortho</i> *	C	10.7	6.77	6.59	7.8
A14 <i>meta</i>	C'	10.3	7.15	6.96	7.8
A19 <i>ortho</i>	D	11.4‡	—	—	7.0
A19 <i>meta</i>	D'	—	—	7.15§	8.9
B16 <i>ortho</i>	B	10.6	6.80	6.54	8.1
B16 <i>meta</i>	B'	10.3‡	7.13	7.04	—
		11.8			
B26 <i>ortho</i>	A	11.4	6.59	6.49	8.1
B26 <i>meta</i>	A'	—	—	—	7.0

* The *ortho* (3, 5) and *meta* (2, 6) protons are located *ortho* and *meta* respectively to the phenolic hydroxy group of the tyrosine side chain.

† δ_A , δ_B are the chemical shifts at the acid limit and the alkaline limit of the titration curve respectively (Fig. 5); J is the coupling constant of the tyrosine doublet.

‡ pK' values not determined by computer methods, hence approximate.

§ This resonance did not undergo any appreciable shift with pH.

|| Second titration curve, see Fig. 5.

inverted by irradiation of other nearby doublets and irradiation of E,E' did not cause the collapse of any other doublets in the spectrum. Since E,E' were not inverted in the spin echo experiment they were assigned to two singlet resonances from the C-4 protons of histidines B5 and B10. We were unable to observe these resonances previously (Bradbury *et al.*, 1981) because of peak overlap at pH 9.85 and they will be discussed in detail elsewhere. The C-4 histidine protons were also recently assigned by Cheshnovsky *et al.* (1983) at low pH.

The first step in the assignment of the four pairs of doublet resonances (Table 1) is to compare the spectra of des-(B26-B30)-insulin (lacking tyrosine B26) at pH 11.55 where it is monomeric (The Conformational Study Group, Peking, 1976), with the spectra of monomeric 2-Zn insulin. The tyrosine doublets (A,A') in Fig. 2(a) are absent from Fig. 2(b) and hence these are assigned to tyrosine B26. ^1H n.m.r. resonances of the (3,5) protons *ortho* to the hydroxy group occur upfield of resonances from the (2,6) protons *meta* to the hydroxy group in tyrosine and its simple derivatives (Campbell *et al.*, 1975b) and this has been assumed to apply in this work, although it should be noted that this behaviour may sometimes be reversed in proteins (Snyder *et al.*, 1976). It is noted that there are several other differences between the two spectra, including shifts in the phenylalanine region (F), possibly due to phenylalanine B25 next to the deleted pentapeptide, slight 0.02 p.p.m. upfield shifts of tyrosine resonances (B,B') and an unidentified resonance X, which may perhaps be due to an impurity in the samples. The tyrosine resonances (B,B') are from tyrosine B16 (Table 1), which has undergone a slight change of environment due to the removal of the five end residues of the B-chain, particularly tyrosine B26.

The doublet resonances C,C' in Fig. 3(a) are absent from Fig. 3(b), which is of $\text{C}^{3,\text{A}14}$ -nitro insulin, shown to be nitrated at tyrosine A14 by Morris *et al.* (1970). Thus resonances CC' are assigned to the *ortho* and *meta* proton doublets of tyrosine A14 respectively. Also in Fig. 3(b) was observed a new resonance G and a resonance H which is coincident with H-2 histidine B10. At low pH resonance H, the H-2 and H-4 (EE') resonances of histidines B5 and B10 and the various tyrosine doublets all moved downfield (Fig. 3c). Resonance H arises from the H-2 proton adjacent to the 3-nitro group, which is consistent with (1) its occurrence as a singlet, since *ortho* coupling is removed, (2) its downfield position and (3) its downfield shift with decrease of pH (Figs. 3b and 3c).

The spectrum of $\text{C}^{3,\text{A}14}$, $\text{C}^{3,\text{A}19}$ -bis(nitro)insulin in Fig. 4(b) shows deletion of resonances C,C'

Fig. 2. Comparison of ^1H n.m.r. spectra of 1.73 mM solutions in $^2\text{H}_2\text{O}$ at pH 11.55 and 37°C of (a) 2-Zn insulin and (b) des-(B26-B30)-insulin, from which doublet resonances A and A' are absent

The signal X may be due to an impurity.

(assigned to tyrosine A14) and D,D' as compared with Fig. 4(a). Thus resonances D,D' are assigned to A19 tyrosine *ortho* and *meta* protons respectively. The spectrum of the bis(nitro)insulin at low pH (results not shown) was similar to that in Fig. 3(c) (Ramesh, 1982). Resonances F (δ 7.33, 7.35) present in the unreacted and mononitro derivatives have shifted in the des-(B26-B30)-insulin (Fig. 2b) and in the bis(nitro) derivative (Fig. 4b), and are probably due to phenylalanine protons, possibly phenylalanine B25. Two small resonances (I and J) are observed in the spectrum of the bis(nitro) product (Fig. 4b), but not in the mononitro or native insulin spectra. Resonances G (Fig. 3b) and I and J (Fig. 4b) require further analysis before assignments are possible. The

Fig. 3. ^1H n.m.r. spectra of the aromatic region of $1.73\text{ mM } ^2\text{H}_2\text{O}$ solutions at 37°C of (a) 2-Zn insulin at pH 11.55, (b) $\text{C}^{3,\text{A}14}$ -nitroinsulin at pH 11.55 and (c) $\text{C}^{3,\text{A}14}$ -nitroinsulin at pH 2.75

remaining unassigned tyrosine residue in insulin (B16) must therefore belong to the only unassigned pair of resonances in Table 1, i.e. B (δ 6.59, 6.62) and B' (δ 7.12).

The titration curves of the assigned tyrosine doublets (Fig. 5) were obtained from spectra of the

type shown in Fig. 1, obtained at 26 different pH values between 9.7 and 12.2. Although the data was collected at closely spaced pH intervals the *ortho* (3,5) and *meta* (2,6) doublets were difficult to follow throughout the titration because of the smallness of the changes in chemical shift with pH,

Fig. 4. ^1H n.m.r. spectra of the aromatic region of 1.73 mM $^2\text{H}_2\text{O}$ solutions at 37°C of (a) 2-Zn insulin at pH 11.55 and (b) C^3A^{14} , C^3A^{19} -bis(nitro)insulin at pH 11.55

severe peak overlap and considerable line broadening at the low pH end of the range, due to aggregation to dimer and hexamer (Ramesh, 1982). The pK' values in Table 1 were obtained from the titration curves by computer methods (Carver & Bradbury, 1984) except for A19 *ortho* and B16 *meta* titration curves where approximate estimates were made based on a small number of data points. The B16 *meta* curves showed a second inflexion at high pH and the B26 *meta* curves shifted downfield with increasing pH, instead of upfield as normally observed (Fig. 5).

Discussion

The assignments of the ^1H n.m.r. resonances of the four tyrosine residues in insulin at pH 11.55 (Table 1) show that the tyrosine rings are freely rotating, since each ring gives rise to a pair of doublets from the protons *ortho* and *meta* to the hydroxyl groups (Campbell *et al.*, 1975b). With reduction of pH below 12 there is considerable difficulty in following the shifts of all these doublets (shown in Figs. 1 and 5), particularly in the region of δ 6.6–6.8. However, the titration curve for A14 *meta* is particularly clear (Fig. 5) and

Fig. 5. Titration curves of the n.m.r. resonances of *ortho* (o) and *meta* (m) protons of tyrosines B26, B16, A14 and the *ortho* (o) protons of tyrosine A19 of 2-Zn insulin in $^2\text{H}_2\text{O}$ at 37°C

gives a pK' of 10.3 (10.7 for the A14 *ortho* proton resonance). This normal pK' value is consistent with the location of tyrosine A14 on the surface of the insulin crystal in the monomer and dimer (Blundell *et al.*, 1972). It is therefore very unlikely that the spectrophotometrically determined high pK' value of 11.7, attributed to a possible hydrogen bond, is correct (Morris *et al.*, 1970).

Tyrosine B16 has a normal pK' of 10.3–10.6, consistent with its complete accessibility to solvent in the monomer and its partial accessibility to solvent in the dimer crystal (Blundell *et al.*, 1972). A very interesting feature of the titration curves is that the B16 *meta* curve, but not the B16 *ortho* curve, shows a second inflexion at high pH with a very approximate pK' of 11.8. The protons *meta* to the hydroxyl group are observing the titration of a nearby group in the insulin monomer, probably tyrosine A19 and/or B26 which are about 1.0–1.3 nm (10–13 Å) from tyrosine B16 (Mercola & Wollmer, 1981).

Tyrosine residues A19 and B26 have high pK' values of 11.4, probably because of their limited accessibility to solvent. Thus, in the crystal, tyrosine A19 lies in a shallow surface pocket with only one *ortho* position accessible to solvent and B26 has only one *ortho* position accessible to

solvent (Blundell *et al.*, 1972). The hydrophobic environment of these residues in the crystal is apparently maintained in solution (but see Fujii *et al.*, 1981) and has the effect of stabilizing the uncharged form of the phenolic ring, hence raising its *pK*, as observed. The inaccessibility of tyrosine A19 is confirmed by the approximate constancy of the A19 *meta* resonance with pH.

The titration curves of tyrosine B26 (Fig. 5) have several interesting features. Both the *ortho* and *meta* doublets are observed only above pH 10.75 (see Fig. 1) due to the dissociation of dimeric insulin to monomer (J. H. Bradbury & V. Ramesh, unpublished work). In the dimer the resonances are broadened due to the aggregation and, in the crystal, the phenolic hydroxyl group is buried and also hydrogen-bonded to a water molecule (Blundell *et al.*, 1972). At high pH the B26 *ortho* resonances are readily observed (Fig. 1) and produce a well-defined titration curve (Fig. 5) with a *pK'* of 11.4. By contrast, the B26 *meta* resonances undergo a slight anomalous titration curve (upfield shift with decrease of pH) which is probably a composite of two effects, i.e. a small normal titration shift and the observation of the titration of another nearby titrating tyrosine residue, probably A19 which is about 0.5–0.6 nm (5–6 Å) from tyrosine B26 (Mercola & Wollmer, 1981).

The important interactions of tyrosine residues B16 and B26 that occur at the monomer–monomer interface on dimerization and the interactions of tyrosine residues A14 and B16 at the dimer–dimer interface on hexamer formation were observed in the crystal (Blundell *et al.*, 1972), and underline the importance of the correct assignment of the n.m.r. resonances of these residues. The assignments given in this paper will be used in further studies on the aggregation properties of insulin (J. H. Bradbury & V. Ramesh, unpublished work).

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