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Conformational transmission in proinsulin and its derivatives: A study using H/D exchange

Hooria Younas[a,1], Qurra-tul-Ann Afza Gardner[a,1], Naeem Rashid[a], J. Neville Wright[b], Muhammad Akhtar[a,b,*]

[a] School of Biological Sciences, University of the Punjab, New Campus, Lahore 54590, Pakistan
[b] School of Biological Sciences, University of Southampton, Southampton SO16 7PX, UK

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ABSTRACT

Buffalo proinsulin cDNA was isolated, sequenced and shown to differ from that of its bovine counterpart in six nucleotides. However, at the protein level the predicted sequences of the two species are identical. Buffalo M-proinsulin, containing the initiation methionine, was produced in *Escherichia coli* and purified to give M_r of 8812. Following the replacement of 99% of the exchangeable hydrogen atoms with deuterons a preparation containing 131 D atoms was obtained. Buffer exchange of the latter into a protio medium led to, the immediate release of 109 (±1) D atoms into the medium and the retention of 22 (±1) D atoms in the protein. The slow exchange of these D atoms was studied at 0 °C/pH 2.8. Insulin derived from buffalo proinsulin as well as bovine when deuteriated and buffer exchanged, similarly, gave the retention of 25 (±1) D atoms. The data show that the secondary structure of the insulin core present within buffalo/bovine proinsulin contains 5 (±1) fewer slow exchanging hydrogen atoms than are present in the final hormone. This effect is attributed, predominantly, to the long range influence of the C-peptide, composed of 26 residues, on the insulin core of buffalo proinsulin. In contrast, in the case of human proinsulin, comprising 31 amino acids in the C-peptide, the secondary structure of the insulin core within human proinsulin is closer to that of insulin itself.

1. Introduction

In a seminal study the primary structure of porcine insulin was elucidated by Sanger in the 1950s [1]. It was subsequently found that insulin from all animals contains A and B chains composed of 21 and 30 amino acids respectively and held by two inter- and one intra chain disulphide bonds [2] as shown by the structure of bovine insulin (**5**, without N-terminal M and C-terminal R, Fig. 1). Human insulin differs from bovine insulin at three positions and from porcine, at only one (Fig. 1S). During the 1960s Dorothy Hodgkin's group reported the X-ray structure of porcine insulin at increasingly higher resolutions and culminated in the description of the final structure at 1.5 Å [3]. It was shown, by the latter structure, that the insulin monomer (within a two Zn containing hexamer) has a compact globular structure with a hydrophobic core containing two helices in the A chain (A2–A8 and A13-A19) and one in the B chain (B9–B19); the latter helix may be extended in different crystal forms as well as solution containing helix stabilising agents. Interestingly, in up to 20% acetic acid the tertiary structure of insulin is retained in solution [4 and citations therein], and in low-pH grown crystals it is monomeric with a well ordered structure as found in other crystal forms [5].[2] Also gel filtration results of human insulin in acidic medium (pH 2.5) show that it exists in a monomeric form (Fig. 2S) [6].

Proinsulin, a biosynthetic precursor of insulin, contains a C-chain linking the A and B chains (**1**, without N-terminal M) [7]. Its X-ray structure, the ribbon diagram of which is included in our

Abbreviations: M-proinsulin, buffalo proinsulin-containing N-terminal methionine expressed using buffalo proinsulin; cDNA, the amino acid sequence of which is the same as that of the bovine protein; M-insulin-RR, buffalo insulin-containing methionine at the N-terminal and two arginine residues at the C-terminal of B chain; M-insulin-R, buffalo insulin-containing methionine at the N-terminal and one arginine residue at the C-terminal of B chain; C-peptide-KR, the conventional C-chain sequence containing lysine and arginine at the C-terminus; R-C-peptide-KR, the conventional C-chain sequence containing lysine and arginine at the C-terminus and arginine at N-terminus; H/D, hydrogen/deuterium; hydrogen, denotes all the isotopes of this element while protium and deuterium are specific descriptions; 'slow exchanging' hydrogen atoms, D atoms of deuterium exchanged proteins which survive following the immediate transfer of the latter into a protio medium.

* Corresponding author at: School of Biological Sciences, University of Southampton, Southampton SO16 7PX, UK.
E-mail address: ma3@soton.ac.uk (M. Akhtar).

[1] The two scientists have made equal contribution to the work in this paper.

[2] These observations underpin the protocol used in the present paper to study H–D exchange in 0.5 M acetic acid, pH 2.8. Further elaboration on the use of low pH for the study is provided in Section 2.1.1 of our previous paper [8].

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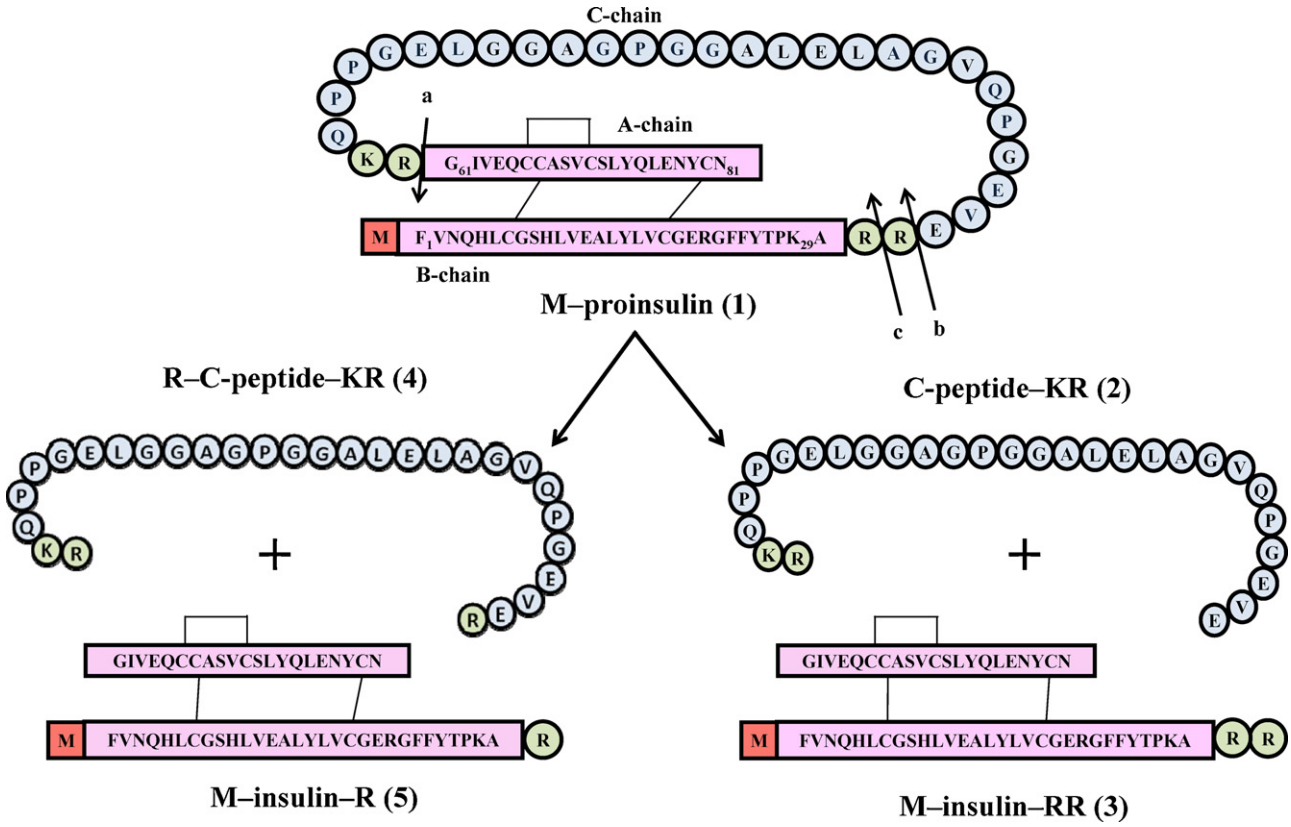

Fig. 1. The sequences of recombinant buffalo M-proinsulin (1) and its tryptic products C-peptide-KR (2), M-insulin-RR (3), R-C-peptide-KR (4) and M-insulin-R (5). The arrows a, b and c show cleavages at R_{60}–G_{61}, R_{32}–E_{33} and R_{31}–R_{32} bonds respectively. The proinsulin numbering system is used above and the N-terminal methionine is considered as residue zero.

earlier paper [8] from an unpublished work [9], lacks necessary resolution in and around the C-chain domain but in general shows that the C-chain is largely unstructured while its insulin moiety has a similar structure to that of the mature hormone. This conclusion is similar to that deduced by the systematic studies of Weiss and coworkers using NMR [4] which has recently led to the description of finer details of the molecule and in particular of the structure of the C–A junction [10]. Some aspects of these findings are compared with the results of H–D exchange experiments described in this paper and reported elsewhere for the human system [8].

Amide hydrogen atoms of unstructured peptide bonds exchange rapidly with the protons of the medium. However, when these are engaged in the formation of secondary structure elements of proteins, the N–H hydrogen atoms may be protected from exchange, the degree of this, besides the temperature and composition of the medium, depends on the extent to which these are buried in the interior of protein, and also on the strength of the hydrogen bond. These exchange-retarded N–H bonds are termed 'slow exchanging' hydrogen bonds and can have a spectrum of exchange half-lives [11–16]. This feature, following the pioneering work of Linderstrøm-Lang [11], has been exploited for the study of protein dynamics and the elucidation of protein secondary structure [15–18].

Using recombinant human proinsulin, and its derivatives, in which all the exchangeable hydrogen atoms had been replaced with deuterons, we have recently shown by H/D exchange technique that its C-peptide domain, comprising 30 peptide bonds, is relatively unstructured, containing only four slow exchanging hydrogen atoms [8]. While, the domain containing the A and B chains of insulin has 23 (±1) slow exchanging hydrogen atoms and has very similar exchange kinetics as found for mature human insulin [8]. A similar conclusion was also deduced by NMR studies [4,10]. We have now extended the H/D exchange study to buffalo proinsulin, whose cDNA, though, differs from that of bovine in six nucleotides, has the same amino acid sequence [19] as bovine proinsulin [20] (Fig. 1S). The C-peptide of bovine/buffalo proinsulin has five fewer amino acid residues, compared to human proinsulin [21] and the work described in this paper shows that this difference has profound influence on the secondary structure of the C-peptide and of the insulin moiety contained within buffalo proinsulin.

2. Experimental

2.1. Preparation of recombinant buffalo M-proinsulin

Total RNA was isolated from the pancreatic samples of Ravi breed of buffalo (*Bubalus bubalis*) by acid guanidinium thiocyanate-phenol-chloroform extraction method [22]. The cDNA of buffalo proinsulin was synthesized by reverse transcription polymerase chain reaction (RT-PCR) using RNA as a template and gene specific primers designed from the published proinsulin mRNA sequence of *Bos taurus* (Accession No. M54979), that is the closest species to *B. bubalis*. A *Nde*I and *Hin*dIII restricted fragment of proinsulin cDNA was ligated into pET-21a vector and the protein produced as inclusion bodies in *Escherichia coli* BL21 – CodonPlus (DE3) – RIL cells. The inclusion bodies were solubilized and reduced in buffered urea, pH 8.0 (8 M urea, 50 mM glycine, 100 mM Tris–Cl and 1 mM EDTA) containing 2 mM DTT and diluted in pulses in refolding buffer, pH 9.0 (100 mM Tris–Cl, 2 mM EDTA, 0.5 mM cystine, 5 mM cysteine, 0.1 mM phenyl methyl sulphonyl fluoride and 1.12 M urea) (final concentration of urea was 2 M) to obtain properly refolded protein. The refolded protein was purified to homogeneity by RP-HPLC using Biobasic C18 preparative column where M-proinsulin eluted in 37% acetonitrile using a gradient made from 0.1% triflu-

Table 1
Molecular masses of protio- and deuterio-M-proinsulin and their tryptic products.

Species	Molecular mass (Da)		No. of D atoms incorporated		% deuteriation
	Theoretical	Experimental	Theoretical	Experimental	
Protio species					
M-proinsulin	8812.0	8812.0			
Singly cleaved M-proinsulin	8830.0	8830.0			
M-insulin-RR	6177.1	6177.0			
M-insulin-R	6020.9	6021.0			
C-peptide-KR	2670.9	2670.8▲			
R-C-peptide-KR	2827.1	2826.9▲			
Bovine insulin (standard)	5733	5733.2▲			
Deuterio-species					
M-Proinsulin	8945.8	8944.1	133	131.4	98.8
Singly cleaved M-proinsulin	8965.8	8964.0	135	133.2	98.7
M-insulin-RR	6274.7	6272.9	97	95.1	98.0
M-insulin-R	6113.4	6111.4	92	90.0	97.8
C-peptide-KR	2711.1	2711.1	40	40.0	100.0
R-C-peptide-KR	2872.4	2871.2	45	43.9	97.6
Bovine insulin (standard)	5819.9	5819.8	86	85.8	99.8

Masses of the protio species were determined by MaxEnt using nanospray on the LCT, while those marked as ▲by manual calculation using Eq. (1). The masses of the deuterio-species, in ^2H_2O, were calculated from the *m*/*z* values of multiply charged ions, obtained by syringe pump injection on the Quattro, and calculated using Eq. (2). The theoretical molecular masses of deuterio-species were calculated by assuming that all the exchangeable hydrogen atoms of the species have been replaced by deuterium. The experimental D content was obtained by subtracting the protio mass from the deuterio mass and dividing by 1.0056 (the difference of atomic weights of D and H). The figures have been rounded to one place of decimal. The calculations of the theoretical exchangeable hydrogen atoms in M-proinsulin and its tryptic products are given in supplemental data. All values above are average masses, since in the mass range 5000–9000, the monoisotopic species containing all ^{12}C atoms will be less than 10% of the total.

oroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile [19].

2.2. Tryptic digestion of protio-M-proinsulin

M-proinsulin (975 μg) was dissolved in 400 μl of 50 mM Tris–Cl, pH 8.0. An aliquot (30 μl, 75 μg) of latter was mixed with 10 μl (0.8 μg) of trypsin, incubated at 20 °C for 5 min, when milky appearance was seen which disappeared following the quenching of the reaction mixture with 3 μl of glacial acetic acid. The M-proinsulin and its tryptic digest were analyzed on Liquid Chromatography Time of Flight Instrument (LCT, Micromass, now Waters) using nanospray as described below (section 2.6).

2.3. Deuteriation of M-proinsulin

HPLC purified buffalo M-proinsulin (2.6 mg/ml) in 50 mM Tris–Cl buffer pH 8.0 was freeze dried. The residue was dissolved in 2 ml of ^2H_2O and kept at 37 °C for 30–40 min and re-freeze dried. The process repeated two times more. The residue was then finally dissolved in 1 ml of ^2H_2O to give 0.3 mM fully deuteriated-M-proinsulin in 50 mM Tris–Cl buffer. An aliquot of the deuteriated M-proinsulin solution was analysed by mass spectrometry for the determination of the deuterium content and the remaining used for tryptic digestion.

Alternatively, 5 ml of 50 mM Tris–Cl buffer pH 8.0, was freeze dried, the residue dissolved in 2 ml of ^2H_2O, and the solution freeze dried again. The residue was then dissolved in ^2H_2O (0.5 ml) and 24 μl of the latter added to an aliquot of 120 μl (288 μg) of M-proinsulin, that had also been HPLC purified, freeze dried and dissolved in ^2H_2O previously. The solution contained 0.23 mM deuteriated-M-proinsulin in 83 mM Tris–Cl buffer. The mixture was incubated at 37 °C for 30 min and stored at −20 °C for future use.

The other samples (bovine insulin, buffalo insulin and different mixtures of these proteins) were also deuteriated using the above methods.

2.4. Tryptic digestion of deuterio-M-proinsulin

Deuterio-M-proinsulin (1.3 mg) in ^2H_2O (0.5 ml) containing 50 mM Tris–Cl buffer, pH 8.0, was mixed with trypsin (30 μg; in ^2H_2O 15 μl), the reaction mixture incubated at 23 °C for 10 min and then terminated by acidification with $C^2H_3COO^2H$ (15 μl). An aliquot was analysed by mass spectrometry on a VG Quattro II as described below (section 2.6) to determine the extent of deuteriation.

2.5. H/D exchange of deuteriated samples

Aliquots of 30 μl of the deuteriated samples were exchanged into 0.5 M acetic acid (pH 2.8) in water by the spin column method. For this purpose, ice-cold slurry (0.7 ml) of Bio-gel P-2, fine (Bio-Rad) in 0.5 M acetic acid in H_2O was placed in an empty spin column, pre-cooled to −20 °C, and centrifuged at 3500 *g* for 3 min. A 30 μl aliquot of the solution to be exchanged was applied to the packed column which was centrifuged at 3500 g for 2–3 min and the pass-through (about 30 μl) collected in a 1.5 ml Eppendorf microcentrifuge tube that had also been pre-cooled to −20 °C and the exchanged material immediately stored on ice and analyzed by mass spectrometry at different time intervals to determine the loss of slow exchanging deuterium.

2.6. ESI-mass spectrometry on LCT using nanospray and VG Quattro II with syringe injection

Samples of protio proteins and of H/D exchanged samples were analysed on LCT using nanospray in borosilicate capillaries, in-house sputter coated with gold. These were filled with the protein sample (4 μl; 4–10 μg) in 0.5 M acetic acid, using a fine micropipette-tip followed by centrifugation. Spectra were accumulated over 80 scans at capillary voltage 1100–1200 V, sample cone voltage, 35 V, extraction cone voltage, 8 V, and source temperature of 53 °C. The time elapsed between removal of the sample and recording of its spectra was from 5 to 7 min.

The multiply charged spectra were processed either by MaxEnt software or manually by Eq. (1) when the values by the two methods agreed within 0.5 Da.

$$M = (m \times z) - (z \times 1.0078) \quad (1)$$

(*M* is the mass of the protein, *m* is the *m*/*z* of the charged species and *z* is the number of charges).

Table 2
Time course of exchange of deuterium from M-proinsulin and its tryptic products.

Analysis time (post-exchange) (h)	M-proinsulin* (8812)	M-insulin-RR* (6177)	C-peptide-KR* (2671)	M-insulin-R* (6021)	R-C-peptide-KR* (2827)
0	22	22	2	23	2
0.5	21	21	1	23	1
1	20	20	1	21	1
1.5	19	20	1	21	1
2	18	18	1	20	1
3	18	17	0	18	0
5	15	16	0	17	0
6	16	14	0	16	0
10	15	14	0	14	0
24	11	10.75	0	12	0
48	9	8	0	9	0
S.E.M. (±)	0.8	1.1	0.4	1.13	0.35

Following the treatment of deuterio M-proinsulin with trypsin the reaction mixture was exchanged into protio medium at 0 °C and samples removed at various time intervals for mass spectrometric analysis. Masses on the top of the columns, in asterisk (*), were obtained after quenching the 0 time sample with 50% formic acid to ensure that there was no contamination of the exchange sample with ^2H_2O. That this, indeed, is the case is shown by the fact that values of the formic acid treated samples (*) are the same as for the corresponding protio species in Table 1. The values in the table are of three time-course experiments and the 0 time value was validated in several additional experiments.

For the analysis of deuteriated proteins in ^2H_2O, analysis by nanospray was found unsatisfactory, presumably due to back exchange of deuterons with protons which may occur in the atmospheric pressure compartment of the instrument, giving lower extent of deuteriation than expected [8]. Analysis was, therefore, performed on a VG Quattro II (now Waters, Manchester, UK), equipped with an electrospray ionization source, using syringe pump as described previously [8]. Aliquots of the protein (50 μl) in 0.5 M $C^2H_3COO^2H$ in ^2H_2O, were injected at a rate of 10 μl/min. Under these conditions, unwanted exchange was avoided by flooding the system with deuterons, and satisfactory extent of deuteriation was obtained from multiply charged ions using Eq. (2), in which, the mass of H^+ in Eq. (1), replaces that of D^+.

$$M = (m \times z) - (z \times 2.0135) \quad (2)$$

2.7. Ensuring that the deuterio-to-protio exchanged samples were free from contamination of ^2H_2O and calculation of the D content of the exchanged protein

It was necessary to ensure that the peaks assigned to deuterio-species, in the H/D exchanged sample, genuinely arose from 'slow exchanging' deuterons in protein and not due to fast exchange by contaminant ^2H_2O (leached during the spin column operation). For this purpose, an aliquot from the sample in 0.5 M acetic acid, was routinely, mixed with an equal volume of 98% formic acid, which denatured the proteins, exchanging all the deuterons with protons, and shifted the peaks to masses corresponding to those of the protio isotopomers. It should be emphasised that by the time the present study was undertaken, our handling of the exchange technique, by spin column, by and large, had been refined to the extent that exchanged samples did not contain, any contamination of ^2H_2O, and masses of all the species, within a fraction of a Dalton, corre-

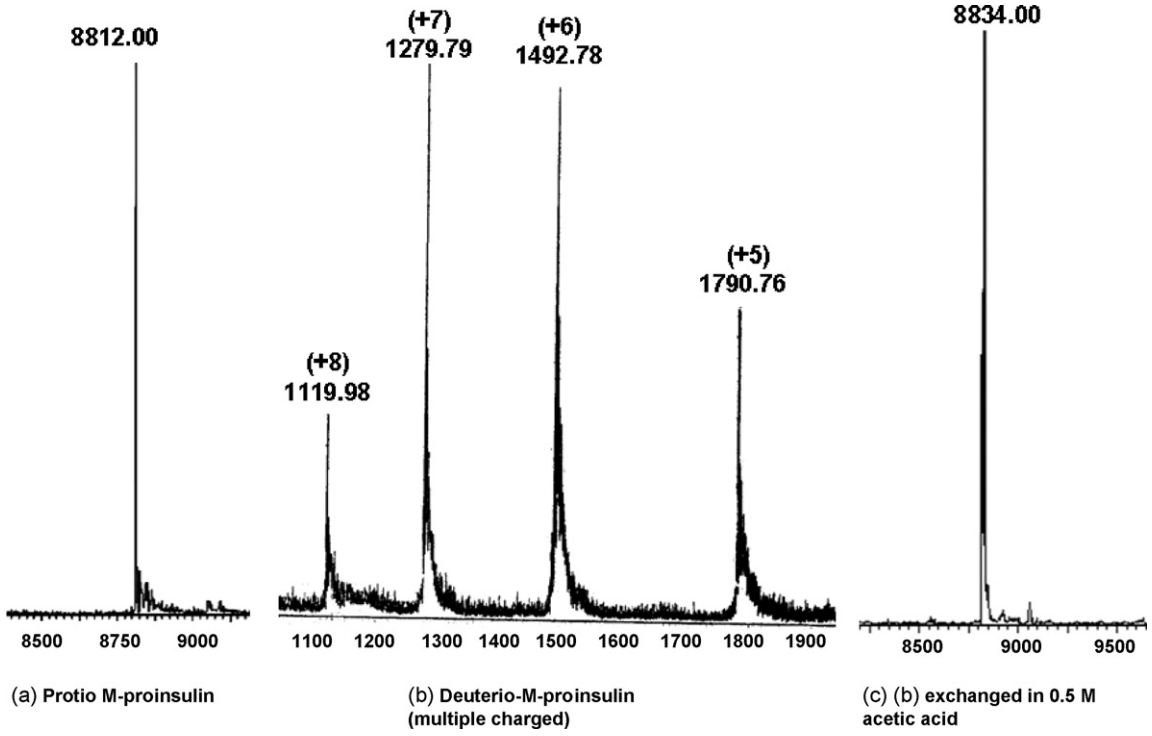

Fig. 2. Mass spectra of (a) protio-M-proinsulin (deconvoluted) (b) deuterio-M-proinsulin (multiple charged) that contains ions at *m/z* 1119.98 (+8), 1279.79 (+7), 1492.78 (+6) and 1790.76 (+5) which gave molecular mass of 8944.1 Da, calculated as described in section 2.6. (c) and (b) exchanged with 0.5 M acetic acid at 0 time (deconvoluted).

sponded to those of the protio species (compare the masses on top of the columns in Table 2 with the masses of the corresponding protio species in Table 1). In the event the formic acid treated samples showed contamination of ^2H_2O, such experiments were discarded, and not processed any further.

The number of deuterium atoms present in a protein, following deuterio-to-protio exchange, is merely the difference of the mass of the protein at that time *minus* that of the formic acid treated sample (this, in the present set of experiments, being identical to that of the protio species). For example, in Table 2, column 2, the 0 time D content of 22 in the recovered M-proinsulin is: 8834 (mass of the exchanged sample at 0 time) *minus* 8812 (mass of the formic acid exchanged sample which is identical to the mass of the protio species in Table 1 (for spectra see Fig. 2a and c)).

3. Results

3.1. Preparation of buffalo proinsulin, its deuteriation and H/D exchange

RNA was isolated from buffalo pancreas, collected from a local slaughter house, and served as template for the preparation of proinsulin cDNA using reverse and forward primers corresponding to two termini of bovine proinsulin cDNA (Accession No. M54979). Nucleotide sequence analysis revealed that buffalo cDNA is composed of 246 base pairs with a coding region for 81 amino acid protein. The nucleotide sequence was found to be 97.5% identical with bovine proinsulin, but at the protein level buffalo and bovine amino acid sequences were identical (Accession No. AB234871). Following appropriate manipulations the cDNA was cloned in pET-21a vector and the protein, containing an additional methionine at the N-terminal, was produced in the form of inclusion bodies. The latter were solubilized in urea and subjected to oxidative renaturation to give after purification refolded protein, denoted as buffalo M-proinsulin (Structure **1**, Fig. 1), which by mass spectrometric analysis was found to have M_r of 8812.0 (predicted from DNA sequence, 8812.0) (Fig. 2a; Table 1).

The exchangeable hydrogen atoms of buffalo M-proinsulin were replaced with deuterons by repeated exchange in ^2H_2O at pH 8.0. The resulting protein was analysed in the Quattro mass spectrometer by syringe pump injection and multiply charged ions were manually processed by Equation 2 (Experimental section 2.6), to allow for the fact that the charges under our analytical conditions are due to $^2H^+$, and not H^+ as underpins various deconvolution algorithms. The calculations gave M_r of 8944.1 (Fig. 2b) for the fully exchanged buffalo proinsulin showing the incorporation of 131.4 deuterons corresponding to 98.8% deuteriation (for the calculation of exchangeable H in proinsulin and its derivatives see Tables 1S and 2S).

The discrepancy 1.2 D atoms between experimentally determined and calculated values, for deuterium exchanged M-proinsulin (Table 1), or for that matter of 0.2 D atoms in insulin, may be attributed to back exchange during various manipulations; in particular, during the exposure of the sample to atmospheric pressure region of the instruments. This facet has been dealt with in Section 2.1, and footnote 1 of our previous paper [8]. There, attention is drawn to the fact that by determining the extent of deuteriation using syringe pump, the back exchange is reduced to within ±1 atom of the theoretical value. Our protocol for the determination of the extent of deuteriation, presents a considerable improvement over 91% deuteriation achieved for an unstructured peptide [18]. Nonetheless, if as a worst case scenario it is assumed that the deficiency of 1–1.5 D atoms, between calculated and experimental values, is due to the protection of this number of hydrogen atoms, from exchange, in the first instant, this would not alter the main message of the work and applies equally to M-proinsulin and the derivatives to which it is being compared. In absolute terms such an event would increase the number of slow exchanging hydrogen by 1–1.5 atoms.

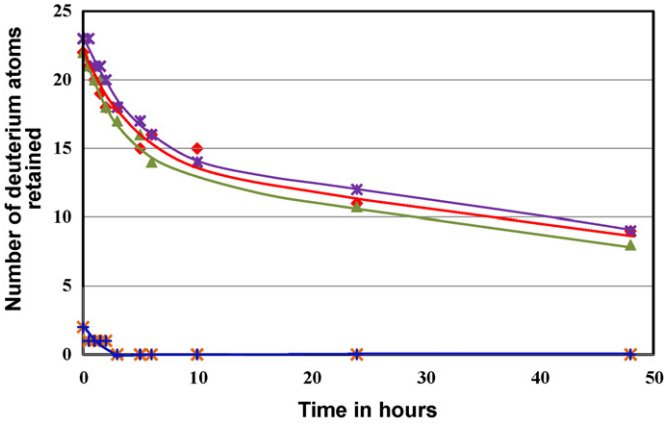

Fig. 3. Time course of H/D exchange. The retention of deuterium atoms from M-proinsulin (1) [———◆———], M-insulin-RR (3) [———▲———], M-insulin-R (5) [———✱———], C-peptide-KR (2) [———✕———] and R-C-peptide-KR (4) [———+———]. The data are from Table 2.

When the fully exchanged deuteriated species, $[^2H_{131}]$M-proinsulin, was exchanged into a protio medium comprising 0.5 M acetic acid, at 0 °C, at a stroke 109 (±1) deuterons, present in unstructured peptide bonds and side chain of polar groups, were washed out and the resulting protein contained 22 (±1) D atoms (Fig. 2c). Time course of these slow exchanging hydrogen atoms was monitored when 8 (±1) deuterons were retained in the protein after 48 h. In order to probe the location of these slow exchanging hydrogen atoms, $[^2H_{131}]$M-proinsulin was treated with trypsin under carefully defined conditions, to produce two sets of cleavage products. These arose from scission, at the dibasic resides involving the C-A and B-C junctions to give the C-peptide as C-peptide-KR and the globular core as, M-insulin-RR (structures **2** and **3** respectively, in Fig. 1). The second set was formed in an analogous fashion but where the cleavage at the B-C junction had occurred between the two arginine residues resulting the formation of the R-C-Peptide-KR (**4**) and M-insulin-R (**5**). Following buffer exchange of the fully deuteriated versions of these species into 0.5 M aqueous acetic acid the kinetics of the loss of deuterons from these species was studied (Table 2; Fig. 3). Both the C-peptide fragments contained only two D atoms, immediately after transfer to the protio medium, which were washed out in the next 2 h. The two globular cores, M-insulin-RR and M-insulin-R, initially had 22 and 23 D atoms respectively, which in a time dependent process were replaced with protium, leaving 8–9 deuterons after 48 h (Fig. 3). The trypsin digestion conditions were so designed that in the same 'pot' were present, in addition to the aforementioned species the uncleaved M-proinsulin. The exchange profile of all these species was very similar.

3.2. Comparison of the H/D exchange profiles of buffalo proinsulin with insulin

In broad terms the exchange profile obtained here for buffalo M-proinsulin and its derivatives is reminiscent of that we have previously reported for human M-proinsulin [8] but there are subtle differences between the two systems. Across species 2–5 more deuterons are retained, immediately after deuterio-to-protio exchange, in human M-proinsulin and its derivatives compared to those of buffalo. These differences needed critical study and the

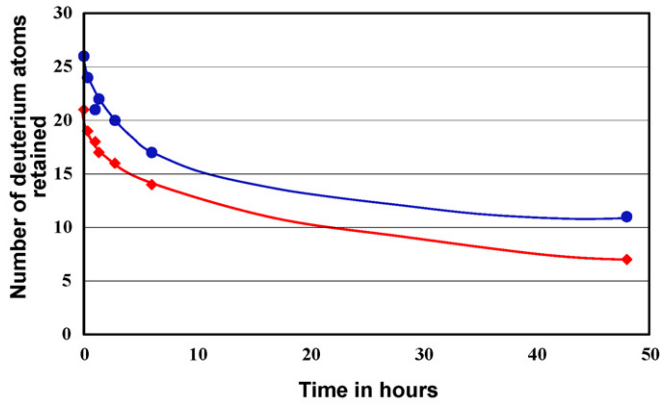

Fig. 4. Comparison of H/D exchange profile of buffalo and human M-proinsulin. Buffalo M-proinsulin [ ] and human M-proinsulin [ ].

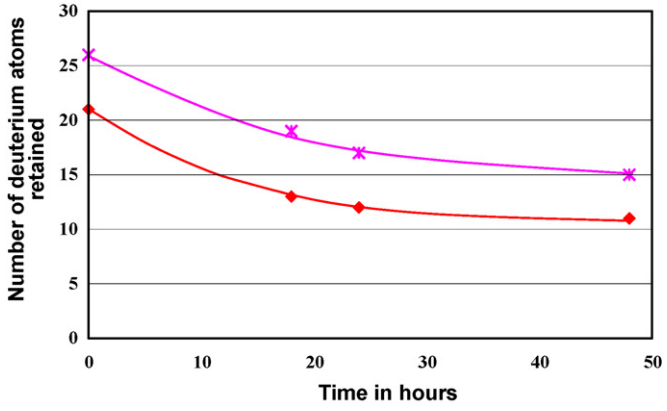

Fig. 5. Comparison of H/D exchange profile of buffalo M-proinsulin and bovine insulin. Buffalo M-proinsulin [ ] and bovine insulin [ ].

following experiments were performed. The fully deuteriated mixture of human and buffalo M-proinsulin in a single-pot was buffer exchanged in 0.5 M acetic acid and the time course of the exchange of deuteron with protium monitored. The profile in Fig. 4 shows that immediately following exchange the human M-proinsulin had retained 5 (±1) more deuterium atoms than its buffalo counterpart, however, the subsequent loss of deuterons from the two species followed a parallel course and at 48 h the initial difference was nearly maintained. Next, the exchange kinetics of fully deuterium exchanged buffalo M-proinsulin and standard bovine insulin (Sigma) were compared and Fig. 5 shows that again buffalo M-proinsulin had lost 5 more deuterons than insulin, thus showing that the secondary structure of the insulin core within buffalo M-proinsulin is populated with 5 (±1) fewer,[3] or weaker hydrogen bond than are present in mature bovine insulin. In order to ensure that the anomalous behaviour of buffalo M-proinsulin was not due to errors in refolding, the protein was digested with V8 protease and cleavage profile expected from correctly folded proinsulin [23,24] was observed (Fig. 3S). Furthermore M-proinsulin was converted into M-insulin which had the same glucose lowering potency as standard bovine insulin [19].

[3] Immediately after exchange, M-proinsulin retains 22 (±1) D atoms, however, 2 of these are attributed to be present in the C-peptide domain. This leaves 20 (±1) D atoms which are assigned to the insulin core *within* M-proinsulin and may be compared to 25 (±1) slow exchanging D atoms found in buffalo/bovine insulin.

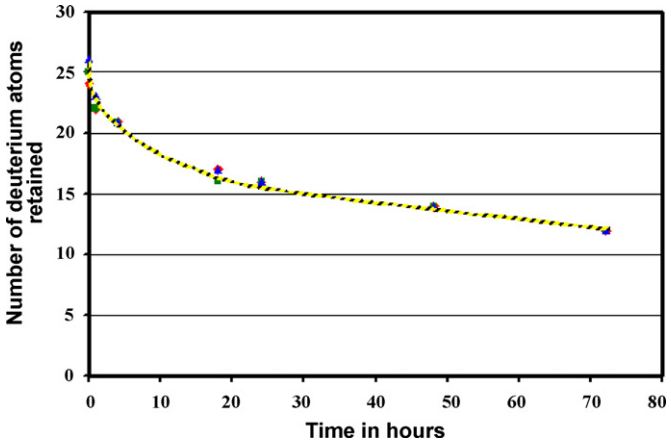

Fig. 6. Comparison of H/D exchange profile of human, bovine and buffalo insulin. Human insulin [ ], Bovine insulin [ ] and buffalo insulin [ ].

More importantly, however, buffalo insulin prepared from an appropriate buffalo proinsulin derivative when fully exchanged with deuterium and buffer exchanged into 0.5 M acetic acid retained 25 (±1) D atoms whose exchange profile was nearly superimposable on the profile obtained with standard bovine insulin or human insulin (Fig. 6). In passing it should be mentioned here, that in an earlier study, Tito *et al.* [18] reported the presence of 15 (±1) slow exchanging hydrogen atoms in bovine insulin. In the latter work, however, the approach was a different one. Bovine insulin was incubated in ^2H_2O, digested with pepsin and the exchange-protected hydrogen atoms in 19 peptic fragments added up to deduce the aforementioned number. The main focus of the approach thus was *not* to maximise the number of slow exchanging hydrogen atoms but to localize their positions in short sequences. It is to be expected that the –NH bonds in the secondary structures of short peptides will be more prone to exchange, with the hydrogen of the medium, than when such elements are present within a folded protein. The protocol [18] will thus underestimate the slow exchanging hydrogen atoms compared to the one used in the present paper. Here, as soon as the fully deuterium exchanged protein is transferred into a protio medium the inventory of D atoms retained in the protein begins to be taken. It should be emphasised that 24–25 (±1) slow exchanging hydrogen atoms estimated in the present work correspond well to a similar number of NH bonds (A2–A8, A13–A19 and B9–B19), engaged in α helices in bovine, pig and human insulin as found by X-ray crystallography [3].

4. Discussion

Previously, we have used the H/D exchange technique to compare the secondary structure elements of human M-proinsulin with its two cleaved products, the insulin core and the C-peptide [8]. The salient feature of these experiments was that human M-proinsulin contained 27 (±1) slow exchanging hydrogen atoms and of the 30 peptide bonds present in the C-peptide only 4 could be categorised as slow exchanging, hence involved in the formation of some form of secondary structure. The insulin core within human M-proinsulin, contained (27 − 4 = 23) (±1) slow exchanging hydrogen atoms, the exchange kinetics of which were very similar to those of 24 (±1) slow exchanging hydrogen atoms present in mature human insulin [8]. Thus the insulin core in M-proinsulin, in the case of the human protein, contained 1 (±1) fewer slow exchanging hydrogen atoms than are present in mature insulin.

There are more than one hundred species of proinsulin currently known and all these contain the same number of amino acids in the A and B chain of the insulin core. However, the C-peptide

in these proinsulin species has 25–38 residues [25 and citations therein]. Since artificial proinsulin analogues in which the connecting C-peptide has been trimmed down to as few as 3 residues can be refolded and processed as the natural pro-proteins [26], it would appear that the length of the connecting peptide does not significantly affect the secondary structure of the bulk of the molecule. In the present work, however, when buffalo M-proinsulin was fully exchanged with deuterons and then buffer exchanged into aqueous 0.5 M acetic acid, there was an 'at a stroke' loss of 109 (±1) deuterons giving buffalo M-proinsulin which retained 22 (±1) deuterons compared to 27 (±1) in human M-proinsulin, under identical conditions (Fig. 4). When the slow exchanging D atoms in the C-peptide are subtracted from those in M-proinsulin then the insulin moiety within M-proinsulin contains (27 − 4 = 23) (±1) and (22 − 2 = 20) (±1) deuterium atoms in the human and buffalo protein respectively. However, extending the comparison to the number of slow exchanging D atoms in the insulin moiety *within* M-proinsulin to those in insulin itself, it was found that the human proinsulin has, on the average, >90% (23 vs 24) of the slow exchanging D atoms found in the mature human hormone. While in the case of the buffalo/bovine proteins, the corresponding value is 80% (20 vs 25). The data indicate that whereas in the human system the C-peptide does affect, to some extent, the number of slow exchanging hydrogen atoms in the insulin core of M-proinsulin, the effect is significantly larger in the case of the buffalo system when the insulin moiety within its M-proinsulin contains 5 (±1) fewer slow exchanging hydrogen atoms than are present in buffalo insulin.

When buffalo insulin prepared in this study, from a buffalo proinsulin derivative, and bovine insulin (Sigma), was deuteriated and exchanged into aqueous 0.5 M acetic acid, it retained similar number of deuterons, *i.e.*, 25 (±1), and the kinetics of the exchange of the two species in Fig. 6 were comparable to that of human insulin [8]. The comparison highlights that, as expected, from the corpus of knowledge in the literature, at the insulin level the hormone from human and buffalo/bovine are very similar. Therefore, the differences in the number of slow exchanging hydrogen atoms seen at the level of M-proinsulin must be due to the length, and or the physicochemical characteristics, of the C-peptide in the two systems. The affect that the C-peptide exerts on the secondary structure of the insulin moiety, within M-proinsulin, could either be due to the long range transmission of its conformational dynamics, or a consequence of a direct interaction of the C-peptide with the insulin core. However, the latter possibility is made unlikely by the NMR and X-ray data, on proinsulin, which show the C-peptide to be 'out of the way' of the insulin core [unpublished work in Ref. [9] cited in [8] and [10]].

Our experiments are susceptible to an error of ±1 Da, hence with small differences being measured here, the emphasis is not on precise values but on the general trend. In broad terms, the comparison of slow exchanging hydrogen atoms in insulin versus proinsulin, of the two species (human and buffalo), indicate that with the 31 residues connecting C-peptide in human proinsulin, its secondary structure elements closely mimic those of its two parts, the insulin-containing core and the C-peptide [8]. The buffalo proinsulin in which the connecting C-peptide is shorter by five residues (of 26 amino acids), seems to have a destabilizing influence on the insulin core domain of the molecule, leading to weakening, or abolition of 5 (±1) hydrogen bonds. *In situ* produced buffalo insulin derivatives containing Arg at C-terminal (**3** and **5**, Fig. 1) also had fewer slow exchanging deuterium atoms, indicating that proximal residues (Arg-Arg in **3** and **Arg** in **5**) may also make some contribution to the destabilization of the insulin core in these derivatives. As the connecting C-peptide of buffalo M-proinsulin is trimmed there is a progressive increase in the number of slow exchanging deuterium atoms, in the insulin core. For M-proinsulin**[3]** the number is 20; for M-insulin-RR, 22; for M-insulin-R, 23 and for insulin, 25 (Table 2; Figs. 3 and 6).

The small but subtle aspects of the long range and proximal effects unravelled by the experiments described above mirror the recent observations of Weiss and colleagues on a derivative of human proinsulin carrying amino acid substitutions at three positions which maintain the protein in a monomeric state [10]. In the latter work using NMR spectroscopy, it was found that near the C-A junction, residues C27-C31 exhibit helix-related Nuclear Overhauser enhancements (NOEs). In the case of human proinsulin (with wild type sequence) we found 4 slow exchanging hydrogen atoms in C-peptide domain of human proinsulin. The indication from our study that the C-peptide, in human proinsulin, affects the number of slow exchanging hydrogen atoms in the insulin moiety of the prohormone is reflected by the conclusion in reference 10 that the helices in A chain are molten, in which intermolecular fluctuations of atoms is larger as compared to this region in insulin. More importantly, in the present paper we observe that relative to mature insulin, the secondary structure of the insulin moiety *within* proinsulin, is more profoundly affected in the case of the buffalo/bovine proinsulin than it is for the human prohormone. We attribute this affect to the differences in the physicochemical properties, or length, of the C-peptide in proinsulin of the two species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2010.07.020.

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