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Conformational effects in reversed-phase high-performance liquid chromatography of polypeptides I. Resolution of insulin variants[1]

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Abstract

In order to further characterise the role of conformation in the retention behaviour of polypeptides and proteins in reversed-phase high-performance liquid chromatography (RP-HPLC), the chromatographic properties of four different insulins have been studied as a function of temperature (over the range 5–85°C) and column residence time (over the range 10–60 min). The role of the ligand structure was also investigated by comparing results obtained with a *n*-octadecyl (C_{18}) and a *n*-butyl (C_4) ligand immobilised to the same porous silica. Comparative structure–retention–stability relationships were determined from an examination of the influence of temperature on a number of chromatographic parameters including the chromatographic contact area, the affinity constant and the experimental band width. The results demonstrated that variations in temperature can be used to affect significant changes in selectivity between the different insulins despite their very high degree of sequence homology. These observations have permitted specific amino acid residues, and in particular those residues encompassing the region A8–A10, to be proposed to be directly involved in the chromatographic contact area of the insulin molecules. Overall, the analysis of the changes in various chromatographic parameters in response to variation of the amino acid sequence, temperature and other experimental parameters provides a powerful tool to elucidate the structural basis for the interfacial stability and the role of conformation on the retention behaviour of polypeptides and proteins in RP-HPLC.

1. Introduction

Insulin, a polypeptide hormone secreted by the islet cells of the pancreas, has a key role in regulating metabolic processes in several tissues (reviewed in Refs. [1] and [2]). Post-translational processing of proinsulin results in a 51 amino acid polypeptide, consisting of two chains, designated the A and B chains. Two of the four conserved cysteine residues within the 21 amino acid residue A chain participate in the two inter-chain disulphide bridges, whilst the remaining two conserved cysteine residues are involved in a single intra-chain disulphide bond. The B chain consists of 30 amino acid residues, disulphide linked through two conserved cysteine residues to the A chain. In vivo, insulin exists as monomeric, dimeric and hexameric forms. The X-ray

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crystallographic structures of various insulins have been extensively described in the scientific literature (reviewed in Refs. [2] and [3]). X-Ray crystallographic determination of the hexameric form of insulin has revealed a compact structure in which three α-helices, encompassing the amino acid residues A1–A9, A12–A19 and B9–B20, form a core structure with the A chain nestled in the arms of the more extended B chain. Two predominant conformations have been identified for the insulin molecule which differ in the extent of helical content of the B chain (either B9–B20 or B1–B20 are in a helical conformation). Similar structures have recently been reported for monomeric insulins in solution based on two-dimensional nuclear magnetic resonance (NMR) spectroscopy [4–7].

RP-HPLC is used extensively for the analysis of peptides and proteins. However, the mechanism of multiple-peak formation in RP-HPLC with otherwise pure samples and the structural and physicochemical origins of the induction of conformational intermediates by the hydrocarbonaceous ligand still remain a poorly understood phenomenon. Several previous studies have examined a number of experimental parameters which can be used to characterise the interactive behaviour of polypeptides in RP-HPLC (see for example Refs. [8]–[12]). In particular, the influence of column temperature, solvent strength and ligand composition of the sorbent on the interactive conformation and dynamics of polypeptides have formerly been investigated. In the present study, similar chromatographic methods have been applied to the study of the retention behaviour of four mammalian insulins chromatographed with *n*-octadecylsilica (C_{18}) and *n*-butylsilica (C_4) sorbents at column temperatures between 5 and 85°C in the presence of aquo–acetonitrile eluents and gradient times of 30–90 min. These studies expand upon our previous work [10–14] and work of others [15,16] who demonstrated that polypeptides generally interact with *n*-alkyl ligands in a preferred orientation and binding conformation(s), which can be perturbed by changes in environmental parameters such as pH, temperature or the chemical nature of the *n*-alkyl ligand.

2. Experimental

2.1. Apparatus

All chromatographic measurements were performed with a Perkin Elmer (PE) Series 4 chromatograph (Perkin Elmer, Norwalk, CT, USA) utilising a PE ISS-100 autosampler, PE LC-95 ultraviolet–visible spectrophotometer and a PE 7500 professional computer with the 'CHROM 3' software package (PE) installed. All peak profiles were routinely monitored at 215 nm and the data stored on the Winchester disc of the PE 7500 computer and processed simultaneously by a PE LCI-100 computing integrator. Further peak analysis was performed using software routines included in the CHROM 3 program framework. Temperature was controlled by either immersing the column in a thermostatted column water jacket coupled to a recirculating cooler (FTS Systems, New York, NY, USA) or by an ICI TC 1900 column oven (ICI Instruments, Dingley, Australia).

Chromatography was performed with Bakerbond wide-pore *n*-octadecylsilica (C_{18}) and *n*-butylsilica (C_4) columns (J.T. Baker Chemicals, Phillipsburg, NJ, USA) with dimensions of 250 × 4.6 mm I.D. and containing sorbents of 5 μm nominal particle size and 30 nm average pore size. All pH measurements were made with an Orion Model SA520 pH meter (Orion, Cambridge, MA, USA).

Electrospray mass spectroscopic analyses of the polypeptide samples prior to and following the chromatographic experiments were carried out on a Sciex AP111 triple quadrupole mass spectrometer (Sciex, Ontario, Canada) operating under typical scan conditions of m/z 300–2400 in 5 s with a scan step of 0.5 a.m.u. Molecular masses of the charged species were calculated using the Hypermass software from the spectra which were collected in the multi-channel averaging mode at orifice potential settings typically between 50 and 140 V.

2.2. Chemicals and reagents

Acetonitrile (HPLC grade) was obtained from Mallinckrodt (Paris, KY, USA) and HPLC-grade

trifluoroacetic acid (TFA) was acquired from Pierce (Rockford, IL, USA). Water was quartz-distilled and deionised in a Milli-Q system (Millipore, Bedford, MA, USA). Bovine, porcine, ovine and equine insulins were all obtained from Sigma (St. Louis, MO, USA) and were of the highest purity available (>95%).

2.3. Chromatographic and computational procedures

Bulk solvents were filtered and degassed by sparging with nitrogen, and solvent reservoirs were maintained under 75 kPa pressure in a nitrogen atmosphere. Linear gradient elution was performed using 0.1% TFA in water (buffer A) and 0.09% TFA in 65% aqueous acetonitrile (buffer B) over gradient times of 30, 45, 60, 75 and 90 min (i.e. solute column residency times of 10–60 min) with a flow-rate of 1 ml/min. Column temperature was maintained at 5, 15, 25, 37, 45, 55, 65, 75 and 85°C and varied typically less than 1°C. To ensure rapid temperature equilibration, the mobile phase was passed through a 50 cm × 0.13 mm I.D. piece of tubing inserted into the equilibrated column jacket or oven before the column inlet.

The insulin solutions were prepared at a concentration of 0.1 mg/ml in 0.1% TFA (buffer A), whilst the injection size varied between 1 to 5 μg of peptide, depending on the absorbance and peak heights of the individual peptides. All data points were derived from at least duplicate measurements with retention times between replicates typically varying less than 0.5%. Various chromatographic parameters related to the retention behaviour, i.e. the logarithmic capacity factor log $\bar{k}$, the media molar fraction of the organic modifier required to affect the elution $\bar{\Psi}$, the band width as 4 σ_v, the slope S and the intercept log k_0 of the plot of log $\bar{k}$ versus $\bar{\Psi}$ were calculated as previously reported [10].

3. Results

3.1. Retention behaviour of insulin variants

The retention behaviour of four different mammalian insulins from bovine, equine, ovine and porcine sources was investigated. These insulins were selected because their B chains have identical primary structures and only one region of their A chains exhibits sequence diversity, namely at amino acid residues A8, A9 and A10 (see Table 1). Moreover, the sequence of bovine and ovine insulins differ by only one amino acid residue at position A9 (i.e. either Ser^9 or Gly^9, respectively), whilst the porcine and equine insulins also only differ at this single amino acid position (i.e. A9 is either Ser^9 or Gly^9, respectively). The differences in selectivity observed with the C_{18} and C_4 sorbents for the equine, ovine and porcine insulins relative to bovine insulin at a gradient time of 90 min are depicted in Fig. 1 as a function of temperature.

Table 1
Solute physical data

Solute	Sequence	Molecular mass
B chain		
All insulins	FVNQHLCGSHLVEALYLVCGERGFFYTPKA	3400.4
A chains		
Bovine insulin	GIVEQCC ASV CSLYQLENYCN	2340.0
Equine insulin	GIVEQCC TGI CSLYQLENYCN	2354.0
Ovine insulin	GIVEQCC AGV CSLYQLENYCN	2309.9
Porcine insulin	GIVEQCC TSI CSLYQLENYCN	2384.0
	A8 A10	
	= inter-chain disulphide bridge	
	= intra-chain dusulphide bridge	

The aligned sequence of the four mammalian insulins including disulphide bridges.

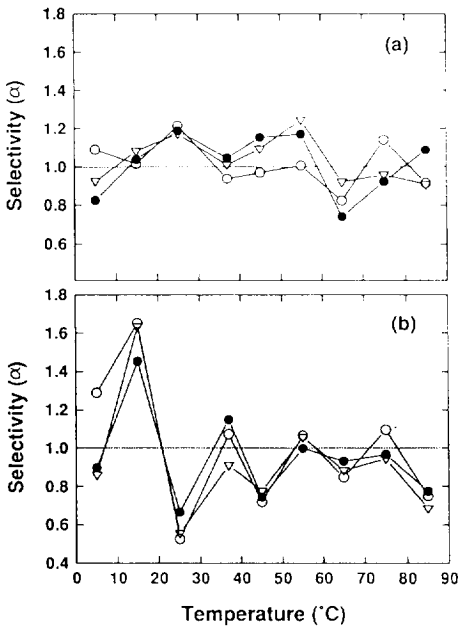

Fig. 1. Plots of the dependency of the selectivity (α) of equine (○), ovine (●) and porcine (▽) insulin with respect to bovine insulin at a gradient time of 90 min as a function of temperature. The insulins were chromatographed with (a) the C_{18} and (b) the C_4 sorbent under the conditions described under Experimental.

The changes in selectivity evident with the two sorbents demonstrate complex retention–temperature dependencies and illustrate that under certain chromatographic conditions all four insulin variants may be resolved. A high degree of resolution was particularly evident at temperatures greater than 45°C with gradient times of 60 and 90 min. As there are only three variant amino acid residues in these four insulin species, the results suggest that amino acid residues A8–A10 either directly participate in the binding site on the surface of the insulin or indirectly contribute to the molecular shape of the binding site by eliciting changes in the conformation of the polypeptide which subsequently affect the molecular geometries of the binding site at the polypeptide–ligand interface. Similar resolution of species variants have been reported for other polypeptides and small proteins in reversed-phase and other interactive modes of HPLC, including cytochrome *c* derived from different species [17], avian lysozyme variants [18,19] and single-site variants of subtilisin [20].

The characteristics of the interactive regions of the insulins can be evaluated from the plots of the logarithmic capacity factor versus the molar fraction of organic modifier, i.e. log $\bar{k}$ versus $\bar{\Psi}$. In particular, analysis of the slope (S value) and intercept (log k_0) values derived from these plots [10] reflect changes in hydrophobic contact area between the solute and the ligands (from S values) as well as the affinity of insulin for the sorbent (from log k_0 values). Least-squares linear regression of the experimental log $\bar{k}$ versus $\bar{\Psi}$ data for all the insulins yielded r^2 values ranging between 0.94 to 1.00 with both sorbents (data not shown). Fig. 2 displays the dependence of the S and log k_0 values on column temperature derived for the insulin variants chromatographed with both the C_{18} and C_4 sorbents. Significant condition-dependent differences in both the S and log k_0 values were observed between each insulin variant. The variations in the S and log k_0 values also correlated with the changes observed in the selectivity between these molecules. Thus, the retention behaviour exhibits differential changes in the interactive contact area and affinity of the insulins as the column temperature was raised from 5 to 85°C. Significantly larger changes in the S and log k_0 parameters were observed with the C_4 sorbent relative to the C_{18} sorbent. These data reflect differences in the mechanism of interaction between insulin and the two physically distinct sorbents [9,10,30,31]. Importantly, in the presence of the C_4 sorbent the retention and selectivity parameters of the different insulins converged at temperatures exceeding 45°C, suggesting that the molecular characteristics of the interaction established by each of the four insulins and the C_4 sorbent became similar at these higher temperatures. In contrast, the data with the C_{18} sorbent displayed a more uniform interactive behaviour as the temperature was elevated between 5 and 85°C. These results suggest that the insulin molecules may adopt more complex interactive structures with the C_4 sorbent at the lower temperatures, which are then perturbed at temperatures exceeding 45°C. These data are

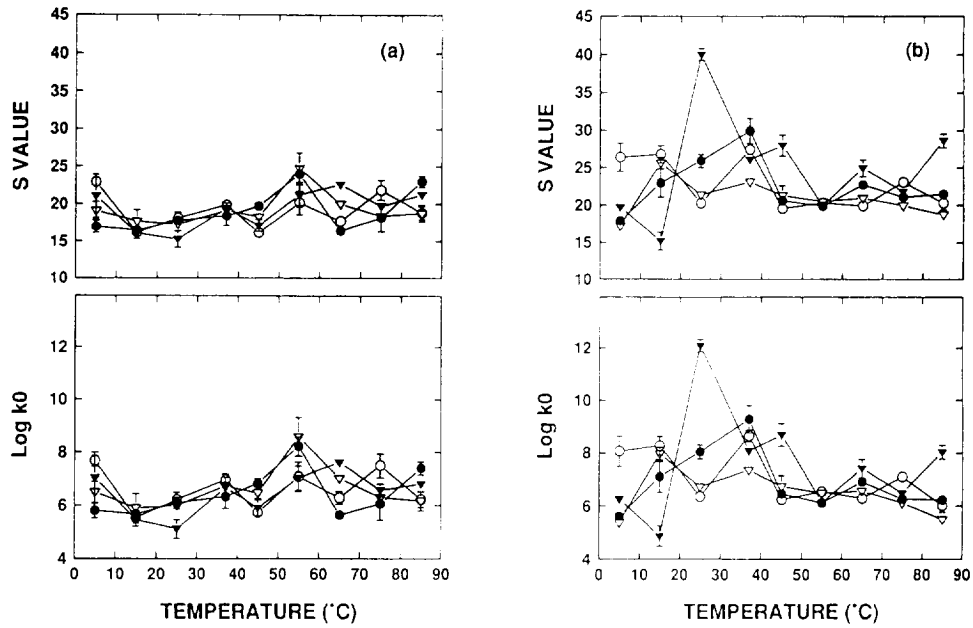

Fig. 2. Dependence of the S and log k_0 values on temperature for equine (○), ovine (●), porcine (▽) and bovine (▼) insulins chromatographed with (a) the C_{18} and (b) the C_4 sorbents.

also consistent with the C_4 ligands acting as a more sensitive probe [10,30,31] to insulin conformation.

3.2. Band width behaviour of insulin variants

The extent to which the influence of secondary equilibria, such as conformational interconversion, can be revealed in the chromatographic profile of a polypeptide depends on the intrinsic efficiency of the system to resolve the different interconverting species and the rate of the interconversion [11]. Broadening of gaussian peaks or the formation of asymmetric, shouldered and multiple peaks with polypeptides and proteins in RP-HPLC are often diagnostic of solute conformational interconversion which can occur over the time scale of the chromatographic migration. The factors that contribute to the intrinsic selectivity of the sorbent to resolve conformational intermediates are dictated by the chemical characteristics of the sorbent surface. In addition, the topographic properties of the different solutes strongly contribute to the intrinsic selectivity characteristics of the system. It can be anticipated that for polypeptide solutes such as these closely related insulins, conformational interconversions that occur following adsorption would result in significant changes in the properties of the interactive region(s) of the solute, due to the ability of small changes in conformation to significantly perturb the molecular geometries which comprise the interactive surface established between the polypeptide and the ligand. Similarly, solution changes in conformation would also affect the associated adsorption kinetics and hence the peak broadening behaviour of these solutes.

The temperature and column residence time dependencies of the experimental band widths derived for the insulins were determined for temperatures between 5 and 85°C and for column residence times between 30 and 90 min duration. Complex elution profiles were observed for the insulins when chromatographed with both sorbents under these conditions. The temperature dependence of the peak shapes of typical chromatograms derived from 90-min gradients for bovine insulin is reproduced in Fig. 3. The bovine insulin peak shapes observed with

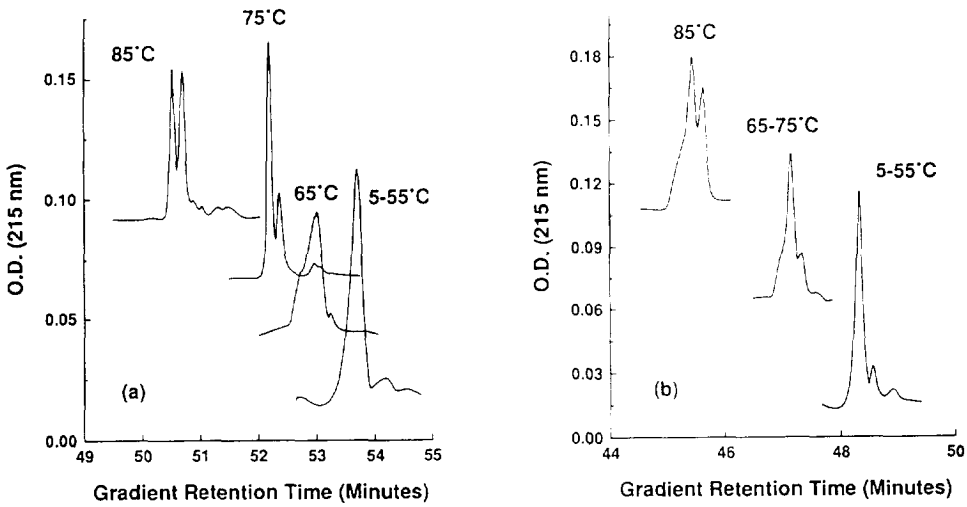

Fig. 3. Elution profiles for bovine insulin chromatographed with (a) the C_{18} and (b) the C_4 sorbents at temperatures between 5 and 85°C.

the C_{18} sorbent were characterised by relatively gaussian peaks between temperatures of 5 and 55°C but with the formation of a broad asymmetric peak at 65°C. At 75°C a second partially resolved peak was observed to be present in the chromatographic profile of bovine insulin for this otherwise pure sample (e.g. reducing and non-reducing gel electrophoresis, electrospray mass spectroscopy and amino acid composition analysis of the purified bovine insulin indicated the presence of a single compositional species). The magnitude of this second peak increased with temperature such that two peaks of approximately equal area were observed at 85°C. These observations suggest that a temperature-induced conformational transition, involving significant changes in the interactive structure of the insulin molecule may be responsible for the observed peak splitting. This behaviour was typical of the other insulin variants investigated, suggesting that whilst the differences in the derived S and log k_0 values observed for these molecules reflected small but nevertheless significant differences in their interactive structure, the overall kinetic processes associated with their interaction with the C_{18} ligand were similar.

Fig. 3 displays representative elution profiles for bovine insulin chromatographed with the C_4 sorbent at gradient times of 90 min. Under these conditions, bovine insulin generated relatively gaussian peaks at temperatures below 65°C. As the temperature increased between 65 and 75°C, a shoulder became evident in the chromatographic profile of bovine insulin which became resolved as a second peak at 85°C. This behaviour was very similar to that observed with the C_{18} sorbent, and suggests that similar interconversion pathways were possibly followed by bovine insulin in the presence of both sorbents.

In order to establish the reversibility of this peak splitting phenomenon, the two peaks observed in the elution profile of bovine insulin chromatographed at 85°C were collected and reinjected separately at 25°C. The two high-temperature fractions had distinct retention times when separated on either sorbent, suggesting that the interconversion between the molecular species responsible for the each peak was irreversible in solution. Similarly, reinjection of the early-eluting peaks collected at 85°C from either sorbent resulted in further interconversion to the later-eluting species when rechromatographed at high temperatures. Thus, it appears that an irreversible change occurred for insulin chromatographed with the C_{18} and C_4 sorbents at temperatures between 65 and 85°C. Each peak

was subjected to electrospray mass spectroscopy which indicated that both peaks correspond to the molecular mass of the parent insulin molecule, i.e. $M^+ = 5733.0$. This result confirms that a conformational change rather than chemical degradation has given rise to the chromatographic profile.

4. Discussion

The RP-HPLC behaviour of the four insulins investigated in this study suggests that the amino acid residues A8–A10 comprise part of the surface region of insulin which interacts with the *n*-alkyl ligands. Moreover, these experimental results support the hypothesis that under certain experimental conditions insulin maintains a folded three-dimensional structure rather than an extended structure during the reversed-phase chromatographic process. Support of this hypothesis firstly comes from the observation that the resolution behaviour for these four different insulins reflected changes in the hydrophobicity of the amino acid substitutions which were all surface-accessible. If insulin was completely unfolded during RP-HPLC, then the more highly conserved hydrophobic core amino acid residues would be expected to dominate the retention behaviour. The investigations of Chu et al. [21], where a conformationally altered human insulin molecule involving substitution of the A chain amino acid residue at position 14 by non-naturally occurring amino acids exhibited large shifts in retention compared to L-α-amino acid analogues which maintained the wild-type conformation, provides further support for this concept. In particular, a single analogue substitution at A-14 (i.e. A chain $Y^{14} \rightarrow$ cyclohexylalanine^{14}) exhibited a very significant increase in relative retention compared to the natural human insulin, suggesting that hydrophobic amino acids exposed due to perturbation of the native conformation and the unfolding of the core of the polypeptide by the bulky cyclohexylalaninyl residue dominated the interaction. In contrast, the four insulins investigated in this study were resolved within a 2-min elution window with a gradient duration of 90 min, clearly demonstrating the incremental amino acid side-chain dependence of retention on the compositional characteristics of the binding region. McLeod and Wood [22] have also provided strong supporting evidence for the maintenance of the insulin tertiary structure during RP-HPLC based on both chromatographic observations and spectroscopic evidence of the behaviour of porcine insulin over a range of pH and solvent conditions. More recently, using near-UV circular dichroism and NMR spectroscopy, Bryant et al. [23] have shown that the structure of human insulin is stabilised in acidic pH conditions, with only minimal changes to the overall structure of the insulin monomer.

Other RP-HPLC studies have achieved similar levels of resolution between highly homologous insulin molecules derived from either chemical modification of the synthetic parent molecule or from different species. In particular, McCleod and Wood [22] have presented data for insulin analogues consistent with our interpretation that amino acid residues A8–A10 form part of the surface of insulin which interacts with the *n*-alkyl ligands. This conclusion is clearly supported by the observation of a relatively large difference in retention between bovine and turkey insulin. The only amino acid residues which differ in these two molecules occurs at residues A8–A10 where significant substitutional changes occur (i.e. Ala[8]–Ser[9]–Val[10] versus His[8]–Asn[9]–Pro[10] respectively). In addition, RP-HPLC studies have also indirectly provided evidence for the involvement of the B chain amino acid residues B27–B30 in the interaction of insulin with *n*-alkyl ligands. For example, RP-HPLC resolution of rabbit, human and porcine insulins [24] and between two variants of mouse insulin [22,25] has been demonstrated. Furthermore, the amino acid residue B25 has also been implicated in the chromatographic contact region, as evident from the resolution between variants of insulin isolated from diabetic patients [24]. Thus, the C-terminal region of the B chain appears to also contribute to the binding site and thus to the chromatographic behaviour of these insulins. It is interesting to note that this

C-terminal segment of the B chain has considerable flexibility in both the crystal state and in solution [23]. Our investigations, as well as the studies by McLeod et al. [26], have indicated that the flexibility of the B chain C-terminus may also be important during polypeptide–ligand interactions in RP-HPLC. For example, the separation of several synthetic insulin molecules involving substitutions or chemical modification at the N-termini of the A and B chains have indirectly implicated the involvement of the amino acid residues A1, B1 and B2 as interactive residues [25]. Based on the results of the present investigation, as well as the above considerations, the location of a preferred contact region for insulins when they interact with *n*-alkyl ligands such as C_4 or C_{18} can be proposed. Shown in Fig. 4 is a space-filling representation of the three-dimensional structure of porcine insulin in which the implicated amino acid contact residues are highlighted in red. As evident from this figure, all of these amino acid residues form a continuous topographic surface.

Analysis of the peak shape of the insulin molecules in terms of their temperature dependence revealed the formation of additional resolvable species between 65 and 85°C. The changes in the elution profile of insulin may be attributed either to conformational changes related to the melting of the tertiary structure of the insulin molecule or to chemical degradation of the solute. The most frequent mechanisms of irreversible polypeptide degradation include cystine destruction, disulphide interchange, cysteine and methionine oxidation, deamidation of asparagine and glutamine residues and hydrolysis of peptide bonds at aspartic acid residues. Fisher and Porter [27] have demonstrated that porcine insulin can undergo two major pathways of degradation under prolonged storage conditions such as one month at temperatures between 5 and 60°C. These degradation processes include deamidation of the glutamine residues at positions A5, A15 and B4, deamidation of the asparagine residues at positions A18, A21 and B3 or polymerisation. More recently, Brange et al. [28] have shown that the asparagine residue at position B3 is particularly sensitive to deamidation. However, in the present study, analysis by electrospray mass spectroscopy of the eluted samples of insulin when chromatographed for relatively short contact exposure times at high temperature demonstrate the presence of minimal degradation products.

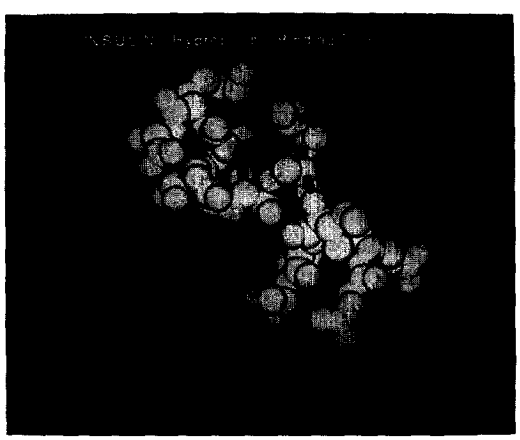

Fig. 4. Computer graphics representation of the X-ray crystal structure of porcine insulin. The peptide backbone of the protein (ribbon-like structure) and the residues A5, A8–A12, B3, B4 and B27–B30 which are proposed to be located in the chromatographic contact region (ball-like structure) are shown.

5. Conclusions

In this investigation, the thermally induced interconversion of insulin was monitored by temperature-dependent changes in the retention parameters, S and $\log k_0$. The differences in these parameters derived for each insulin molecule arose from condition-dependent selectivity changes in the interactive behaviour of the insulin variants with the two different sorbents. The kinetics of the unfolding of insulin was investigated by examining the dependence of the elution profiles on temperature and column residence time. Considerably more complex retention behaviour and elution profiles were observed for these four insulin variants compared

to the retention behaviour of smaller peptidic solutes previously studied under similar conditions [11]. The results of this study with several closely related polypeptides further illustrate the role which differential changes in the microenvironment of the ligand interface, conformation or the molecular composition of the contact region associated the interactive surface of these molecules can have on resolution. Based on these studies, a model can be proposed which is consistent with the involvement of the N-terminus of the A chain, the loop region (residues A8–A12) between the two α-helices of the A chain and segments involving both the N- and C-terminal regions of the B chain all appear to contribute to binding of insulin to the C_{18} and C_4 ligands. These regions are all located within a discrete surface region as evident from computer graphics analysis (see Fig. 4). The present study therefore demonstrates that insulin appears to maintain a significant degree of its folded structure in the reversed-phase environment, with the location of the interactive surface which insulin establishes with the hydrocarbonaceous ligand correlating with a continuous surface structure as revealed in the three dimensional X-ray crystal structure of porcine insulin. In an associated paper [29], the interaction of insulin with reversed-phase sorbents and the nature of the contact region has been further investigated by characterising the individual role of the A and B chains of bovine insulin in this interactive process using similar chromatographic techniques.

Acknowledgement

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