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High-performance liquid chromatography of insulin

Accessibility and flexibility

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SUMMARY

Current ideas suggest that a conformational change in the insulin monomer may play an important part in its interaction with the insulin receptor. An investigation is reported in which analytical reversed-phase high-performance liquid chromatography of insulin analogues was used to investigate the solution conformation of the insulin monomer. The results are interpreted in terms of elution coefficients modified by the calculated surface accessibilities of individual residues. The results suggest a partial unfolding of the insulin monomer under the experimental conditions used, which is consistent with current ideas on the biologically active conformation of insulin.

INTRODUCTION

Recent reports of shortened insulin analogues with either normal or enhanced potency[1–6] have focused attention on the role of the C-terminal region of the insulin B-chain in hormone–receptor interactions. By amidating the terminal carboxylate of B26–B30 despentapeptide insulin (DPI), its biological activity is increased from approximately 20% of that of native insulin[7] to greater than 100%[2–4]. Studies with analogues containing substitutions at B24 and B25 which, in whole insulin, are known either to decrease or to increase biological activity, have demonstrated that the terminal pentapeptide may have an inhibitory or, in some instances, an enhancing role in the biological action of insulin[1–6].

A receptor binding region, centred on B25 Phe, was originally proposed in the 1970s[8,9] and, since then, the concept has been expanded to take account of a possible role for flexibility of the insulin monomer in the expression of its biological activity[10]. A model has been produced which assumes a specific role for conformational flexibility in the C-terminal region of the B-chain[3].

In the work reported here we used analytical reversed-phase high-performance liquid chromatography (HPLC) of a series of modified insulins to assess the possible

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effects of flexibility in the C-terminus of the B-chain on HPLC elution behaviour. The analogues used were DPI, B23–B30 desoctapeptide insulin (DOI), desAsn-A21, desAla-B30 (DAA) insulin and native bovine insulin. The methodology used is an extension of our earlier work on the use of analytical reversed-phase HPLC as a probe of the three-dimensional structure of insulin, where we were able to make a qualitative interpretation of the effects of chemical modification of surface amino acids in terms of insulin's known three-dimensional structure[11].

Earlier attempts to predict the elution of peptides in reversed-phase HPLC, on the basis of their composition, have relied on assigning to each amino acid an elution coefficient based on an experimentally derived estimate of its tendency to affect retention[12–14] and to assign an aggregate coefficient for a peptide based on the sum of the values for its constituent amino acids. This method works well for many short peptides but has been criticized for several reasons, the most important of which are that it assumed an equal contribution from all residues in the peptide and is therefore clearly inapplicable to folded proteins. In addition, the value of the elution coefficient for a particular amino acid has been shown to be affected by its position in a sequence[15].

Here we used the calculated surface accessibility of each residue to modify its elution coefficient, allowing the method to be applied to folded molecules and the experimental result to be interpreted in terms of several modelled conformations. By using analogues which share the same sequence, except where residues are deleted from the ends of chains, we have aimed to reduce sequence-dependent effects. In an earlier examination of the elution behaviour of chemical analogues of insulin, where we did not take account of the surface accessibility of individual residues, we found that it was not possible to explain our results quantitatively and our analysis was therefore based on a qualitative assessment of the chemical nature of the substituents combined with our knowledge of the refined crystal structure of two-zinc insulin[16]. The effects on elution of groups such as acetyl, succinyl and thiazolidine and amino acids such as Arg, Glu and Phe, attached to the N-terminus of the A-chain and, for acetyl, the B-chain, were assessed in terms of the assumption that their chemical environments were determined by the structure of the two-zinc insulin monomer; the experimental results were found to fit well with most of our theoretical predictions. In addition, an examination of the elution behaviour of DAA insulin allowed us to comment on the effects of pH on the solution conformation of this analogue which is considered to be significantly disturbed at neutral pH as compared with native insulin[17] but which, in our study, eluted close to native insulin at pH 2.

In this paper, we interpret our results in terms of several possible conformations of the insulin molecule. The use of analogues with residues deleted from ends of chains and an interpretation which uses the calculated surface accessibility of individual residues together with elution coefficients allow us to estimate how far the conformation of the C-terminus of the B-chain deviates in our system from the two-zinc crystal structure. Several sets of elution coefficients have been used, including three derived from work by Houghten and De Graw[15], who produced ranges of values for each residue type depending on its sequence position.

The basis of our calculations was the refined crystal structure for porcine insulin[16], with the necessary side-chain substitutions to make the sequence compatible with bovine insulin. Fig. 1 shows a stereo view of the insulin monomer. In addition to

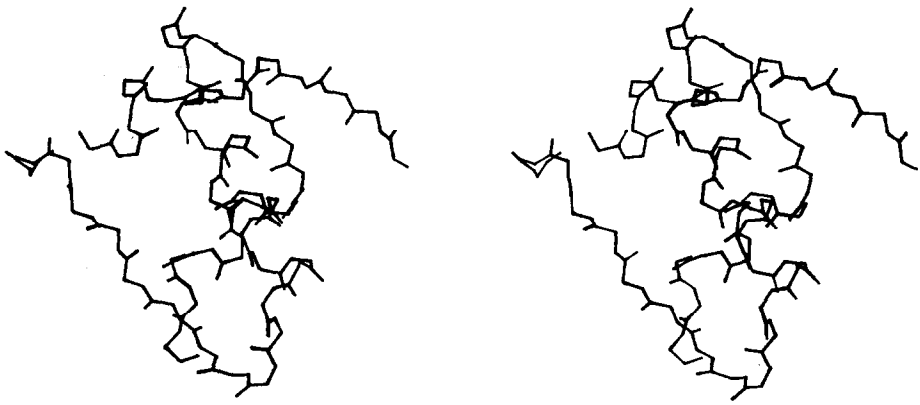

Fig. 1. Stereo view of the insulin monomer main chains (molecule 1, Chinese convention).

calculating accessibilities for the native monomer crystal structure, we have made the necessary deletions to allow us to model DOI, DPI and DAA insulin and have calculated accessibilities for modelled insulins with unfolded B-chain C-termini.

EXPERIMENTAL

Two series of experiments were carried out using a Varian 5000 Series liquid chromatograph equipped with an ODS Ultrasphere column supplied by Beckman. The mobile phase consisted of two components. Solution A was phosphate buffer which, for the first series of experiments, was made up by adjusting a 0.1 *M* solution of sodium dihydrogenphosphate to pH 2 with orthophosphoric acid and, for the second series, consisted of a solution of disodium hydrogenphosphate adjusted, as above, to pH 6.5. Solution B was acetonitrile. All reagents used for solution A were of AnalaR grade, supplied by BDH; water was distilled and deionized. Acetonitrile of HPLC grade 'S' was supplied by the Rathburn. Bovine insulin was supplied by Eli Lilly, DOI was prepared by the method of Bromer and Chance[18] and DPI by the method of Gattner[7]. DAA insulin was prepared by the method of Young and Carpenter[19].

Accessibility calculations and elution coefficients

Solvent contact surfaces for individual residues were calculated by the method of Richmond and Richards[20], which is a development of the method of Lee and Richards[21] for calculation of solvent-accessible surfaces, using computer programs written by S. Islam[a]. This method gives the surface area (Å^2) for each atom which can come in contact with a spherical probe of radius 1.4 Å, taken as being equivalent to a 'standard' solvent molecule. We used the sum of the accessible surfaces for all atoms within a residue as a measure of the contribution which that residue will make to the elution of the insulin molecule as a whole. Elution coefficients were derived from refs. 13–15. The solvent-accessible surface was calculated for each residue in the insulin

[a] Programs written in our laboratory using algorithms from ref. 21.

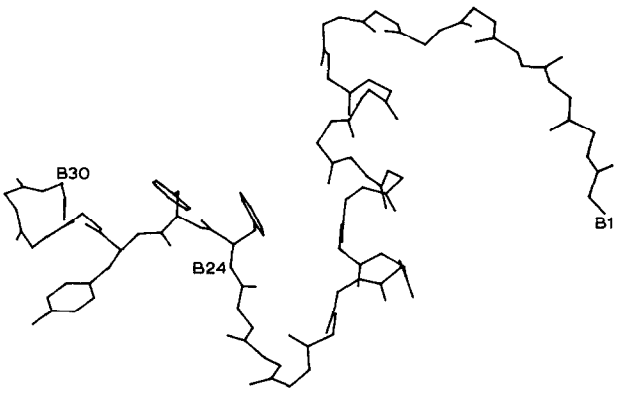

Fig. 2. Insulin B-chain with the C-terminal region unfolded from B24.

molecule. All residues where the surface accessibility of the side-chain alone was less than 7% of the maximum were considered to be buried, according to the criterion of Hubbard and Blundell[22], and eliminated from the calculation. Each of the remaining residues was then considered individually and its accessibility multiplied by its elution coefficient, producing a unique new coefficient for each exposed residue. These coefficients were then summed to give an overall coefficient for the whole molecule which could then be used to calculate its predicted relative elution time.

Coordinates for two-zinc porcine insulin refined at 1.5 Å resolution were extracted from the Brookhaven Protein Databank[16]. As bovine insulin and analogues were used in the HPLC experiments, the coordinates were modified by changing A8 Thr to Ala and A10 Ile to Val. Coordinate sets for DPI, DOI and DAA insulin were produced by deleting the appropriate residues. In addition, three modified coordinate sets were produced. For the first of these, the C-terminal region of the B-chain was unfolded at B23. B23 is a glycine which is conserved in all known insulin sequences and, in the known crystal structures, has main-chain dihedral angles which are inaccessible to L-amino acids with side-chains. It is one of the residues which forms part of the sharp turn which allows the B-chain to turn back upon itself between the

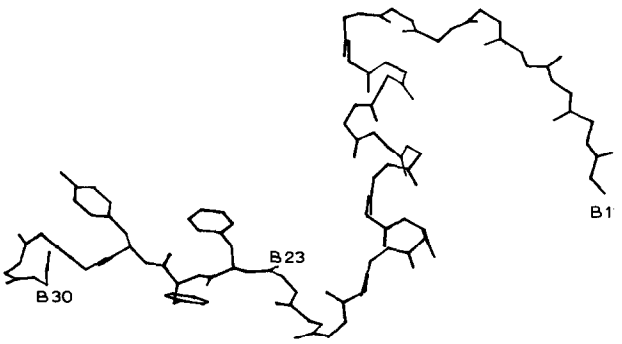

Fig. 3. Insulin B-chain with the C-terminal region unfolded from B23.

B-chain helix and the strand which participates in β-sheet formation across the dimer interface. B23 was seen as a point of significant flexibility and, in the monomer where the β-structure at B24–B26 is no longer present, it is possible that significant changes in dihedral angles may take place. The main-chain torsion angles for B23 are changed from $\varphi = 88.42°$, $\psi = 172.16°$ to $\varphi = 30.16°$, $\psi = -122.63°$, exploring a different area of the conformational space available to a glycine with a positive φ and allowing maximum separation between residues B24 and B25 and the core of the molecule. Fig. 2 shows the conformation of the B-chain in the resulting model. The second coordinate set was made by changing the main-chain torsion angles for B24 from $\varphi = -154.74°$, $\psi = 171.30°$ to $\varphi = -145.02°$, $\psi = -49.88°$, producing a conformation at B25 which is generally similar to that seen in the crystal structure for the monomeric insulin analogues DPI[23]; the B-chain conformation in this model is shown in Fig. 3. The third, which applied to coordinates for DOI only, was produced by changing the torsion angles of B20–B23 to $-140°$, $140°$, maximising the accessibility of these residues. All modifications to the native crystal structure were carried out using the program FRODO[24], running on an Evans and Sutherland PS300 computer graphics system.

RESULTS AND DISCUSSION

Before discussing the results, we shall consider the expected conformation and aggregation state of insulin under our experimental conditions. In solution, insulin aggregates to dimers and, in the presence of zinc, to hexamers. In the experiments described here, all samples were made up at a concentration of 1 mg/ml in phosphate buffer at either pH 2 or 6.5. Under these conditions insulin exists as a monomer–dimer equilibrium with dimers predominating at pH 6.5 and monomers at pH 2 (ref. 8). During the chromatographic process itself, the presence of acetonitrile and/or interaction with the hydrophobic column packing would be expected to interfere with any hydrophobic interaction between monomers. It is known that various organic solvents, such as methanol, dimethylformamide, dioxane and butanol, are capable of causing dissociation of dimers to monomers[8]. The reactivity to iodination of the B-chain tyrosine residues, which is slow in aqueous solution where dimers predominate, is accelerated in the presence of butanol, suggesting that insulin is essentially monomeric under these conditions[8]. We have argued[11] that the general consistency of chromatographic results over a pH range, and the similar elution behaviour of insulins capable of dimerization and insulin analogues such as DOI, which lacks the B-chain residues essential for dimer formation and hence exists in solution as a monomer, suggest that all insulins are monomeric under the range of experimental conditions used in this study. Circular dichroism spectroscopy of native bovine insulin, carried out in 0.1 *M* phosphate containing 28% acetonitrile, also suggest that insulin is monomeric under these conditions[25].

We have previously considered the effects of the experimental conditions on the conformation of the monomer[11]. The most significant effects are likely to be low pH (in experiments carried out at low pH), the presence of organic solvent or the interaction of the insulin monomer with the hydrophobic stationary phase, and we have concluded that the basic conformational framework of the molecule is likely to be preserved under these conditions to the extent that it is possible to explain the effects

on elution behaviour of chemical modifications to insulin in terms of the known crystal structure of the monomer. Considering the effects of interaction between the insulin monomer and the stationary phase, it is accepted that the nature of the solvent-accessible surface determines retention characteristics[26], as it alone defines the variation of the observed partition coefficient. It is also possible that we are observing the relative reversibility of an unfolding process occurring at the hydrophobic surface. If we make the reasonable assumption that the unfolded states have a common framework consisting of the three insulin helices and the extended region of the A-chain whose relative orientations, in the native monomer, are determined by the three disulphide bridges, then the free energy of mass transfer between the phases must be a function of the solution conformation which, in turn, defines accessible surface area.

Conservation of the three-dimensional structure of the flexible ends of the B-chain is less certain, however. In this study, we examine the possible effects on insulin's HPLC elution behavior of conformational changes in the C-terminus of the B-chain.

In the crystal structure of the two-zinc insulin dimer, the major secondary structural elements are three helices in each monomer and the antiparallel β-sheet which is formed across the dimer interface between residues B24, B25 and B26 and their counterparts on the second monomer. This short area of sheet involves four hydrogen bonds which, in the dimer, play a major part in stabilizing the conformation of this part of the B-chain. The situation in the monomer is less clear. In the crystal structure of DPI[23], which is monomeric, there is a significant change in the main-chain dihedral angles of B24, which result in a displacement of B25 from its position in the two-zinc structure. We have, at present, no direct evidence for the conformation of this region of the B-chain in a native insulin monomer in solution.

B25 Phe has a significant place in both the proposed receptor binding region[9] and the region responsible for negative cooperativity in hormone–receptor interaction[27]. It also plays a crucial role in the receptor binding model proposed by Nagagawa and Tager[3], where a change in the conformation of the C-terminal region of the B-chain is a necessary part of the expression of biological activity. It is of great interest, therefore, to have a method for the investigation of the conformational stability of the C-terminal region of the B-chain.

TABLE I

EXPERIMENTALLY DETERMINED RELATIVE ELUTION TIMES WITH RESPECT TO BOVINE INSULIN, MEASURED AT pH 2 AND 6.5

Insulin[a]	*RET*	
	pH 2	*pH 6.5*
DOI	0.56	0.56
Bovine insulin	1.00	1.00
DAA insulin	1.20	2.00
DPI	1.09	0.88

[a] DOI = B23–B30 desoctapeptide insulin; DAA insulin = desAsn-A21,desAla-B30 insulin; DPI = B26–B30 despentapeptide insulin.

TABLE II

PREDICTED RELATIVE ELUTION TIMES FOR THE SAME ANALOGUES AS IN TABLE I, DETERMINED FROM ELUTION COEFFICIENTS[12,13,15] ALONE

Basis	*Insulin*	*RET*
Guo, pH 2	DOI	0.84
	Bovine insulin	1.00
	DAA insulin	0.99
	DPI	0.95
Guo, pH 7	DOI	0.83
	Bovine insulin	1.00
	DAA insulin	0.99
	DPI	0.94
Houghten high	DOI	0.82
	Bovine insulin	1.00
	DAA insulin	0.99
	DPI	0.93
Houghten mean	DOI	0.81
	Bovine insulin	1.00
	DAA insulin	1.00
	DPI	0.95
Houghten low	DOI	0.75
	Bovine insulin	1.00
	DAA insulin	1.07
	DPI	1.06

The elution times of bovine insulin, DAA insulin, DPI and DOI at pH 2 and 6.5 are presented in Table I and there are several significant features. Firstly, DAA insulin elutes close to both native bovine insulin and DPI at pH 2, but its relative elution time (RET) with respect to native insulin is increased from 1.20 to 2.00 at pH 6.5; none of the other analogues behave in this way. Second, DOI elutes significantly before native insulin at both pH values, suggesting that it is much more hydrophilic.

Table II shows the predicted RETs for the native bovine insulin monomer, DOI, DPI and DAA insulin, calculated from elution coefficients alone, with no correction for accessiblity. Five predictions are presented, two based on coefficients from Guo *et al.*[12] for pH 2 and 7, and three based on coefficients from work by Houghten and De Graw[15], where it was demonstrated that each residue type may have a range of elution coefficients based on its sequence environment. These are designated Houghten 'high', 'medium' and 'low', and are derived from the maximum, mean and minimum values for each amino acid type as specified in ref. 15. It can be seen that there is basic agreement between the predictions based on both of the Guo coefficient sets and all three Houghten sets. The Houghten low coefficient set generally gave more variable results than the others in all of our predictions and, for this reason, the following arguments are based on predictions calculated from the other four sets, although all predicted RETs are presented in the tables.

Comparing these predictions with the experimentally determined values, it can be seen that the prediction gives a general idea of the RETs observed experimentally in that the RETs for DPI, DAA insulin and native bovine insulin cluster together at pH 2 whereas DOI appears more hydrophilic. This method fails, however, to predict

TABLE III

PREDICTED RELATIVE ELUTION TIMES DETERMINED FROM ELUTION COEFFICIENTS AND RESIDUE ACCESSIBILITIES[21] CALCULATED FROM THE TWO-ZINC INSULIN MONOMER

Basis	*Insulin*	*RET*
Guo, pH 2	DOI	0.98
	Bovine insulin	1.00
	DAA insulin	1.02
	DPI	1.00
Guo, pH 7	DOI	0.94
	Bovine insulin	1.00
	DAA insulin	1.03
	DPI	1.08
Houghten high	DOI	0.93
	Bovine insulin	1.00
	DAA insulin	1.02
	DPI	1.05
Houghten medium	DOI	0.95
	Bovine insulin	1.00
	DAA insulin	1.03
	DPI	1.14
Houghten low	DOI	1.24
	Bovine insulin	1.00
	DAA insulin	1.26
	DPI	2.25

either the extent of the difference between the observed RET for DOI and the other analogues or the discrepancy between the RET for DAA insulin at pH 2 and 7.

Further predictions were therefore carried out using elution coefficients modified by calculated surface accessibilities of individual residues as described under Experimental. Table III shows the results obtained from accessibility calculations carried out on models based on the conformation of the two-zinc insulin monomer molecule 1 (Chinese convention). It can be seen that all of the RETs are clustered around 1.0, and the fit to the experimental data is worse than predictions based on elution coeffiecients alone. Accessibilities here are calculated for a globular monomer where buried residues make no contribution to the solvent-accessible surface and the remaining residues contribute to the surface, and hence the total chromatographic contact area, depending on their relative exposure. The use of elution coefficients alone assumes that all residues are equally accessible to the solvent, so these results appear to suggest that, under our experimental conditions, the molecules are partially unfolded.

As the region of the B-chain from B24 and B26 is stabilized in the two-zinc dimer by inter-monomer hydrogen bonding, it is probable that its conformation will be different in the monomer. Any unfolding in this region is likely to have a significant effect on the accessibility of the hydrophobic residues B24 Phe–B25 Phe and, therefore, their relative contributions to the overall hydrophobicity of the molecule, combined with an increase in the accessibility of A2 Ile, allowing it to contribute to the surface of the molecule. Modelled coordinate sets were therefore produced, as de-

TABLE IV

PREDICTED RELATIVE ELUTION TIMES DETERMINED FROM ELUTION COEFFICIENTS AND RESIDUE ACCESSIBILITIES CALCULATED FROM THE TWO-ZINC INSULIN MONOMER UNFOLDED AT B24

Basis	*Insulin*	*RET*
Guo, pH 2	DOI	0.82
	Bovine insulin	1.00
	DAA insulin	1.01
	DPI	0.99
Guo, pH 7	DOI	0.79
	Bovine insulin	1.00
	DAA insulin	1.02
	DPI	0.96
Houghten high	DOI	0.84
	Bovine insulin	1.00
	DAA insulin	1.07
	DPI	1.00
Houghten medium	DOI	0.79
	Bovine insulin	1.00
	DAA insulin	1.03
	DPI	1.00
Houghten low	DOI	0.75
	Bovine insulin	1.00
	DAA insulin	1.17
	DPI	1.53

TABLE V

PREDICTED RELATIVE ELUTION TIMES DETERMINED FROM ELUTION COEFFICIENTS AND RESIDUE ACCESSIBILITIES CALCULATED FROM THE TWO-ZINC INSULIN MONOMER UNFOLDED AT B23

Basis	*Insulin*	*RET*
Guo, pH 2	DOI	0.68
	Bovine insulin	1.00
	DAA insulin	1.23
	DPI	0.99
Guo, pH 7	DOI	0.66
	Bovine insulin	1.00
	DAA insulin	1.26
	DPI	0.96
Houghten high	DOI	0.67
	Bovine insulin	1.00
	DAA insulin	1.18
	DPI	0.95
Houghten medium	DOI	0.63
	Bovine insulin	1.00
	DAA insulin	1.20
	DPI	1.00
Houghten low	DOI	0.41
	Bovine insulin	1.00
	DAA insulin	1.30
	DPI	1.30

scribed under Experimental, with the B-chain unfolded from B24 and from B23. These models were then used as a basis for further accessibility calculations and RETs calculated as before. The results are shown in Tables IV and V. It can be seen that these results approach the experimental values more closely, with the B23 unfolded model giving the best fit. It is important to note that the accessibilities for DOI are the same in all instances, the B24 and B25 unfoldings being within the octapeptide which is deleted in this analogue. The hypothesis which best fits these results is that, under the experimental conditions adopted, unfoldings in DPI and native insulins increase their retention times, leading to a lower relative elution time for DOI. This is probably due to the consequent increase in the accessibility of the hydrophobic side-chains of B24 and B25 Phe, together with the partial exposure of core residues such as A2 Ile.

There is some evidence that a change in conformation in the insulin monomer from the two-zinc crystal structure may be a necessary part of receptor binding and expression of biological activity[1–6,10] and a model has been produced which implies a conformational change in the C-terminal region of the B-chain[3]. In addition, visual inspection of the crystal structure for both molecules 1 and 2 suggests that a change in the position of the pentapeptide B25–B30 would improve the accessibility of residues A3, A4 and A5, facilitating their ability to bind to the receptor. The crystal structure for DPI[23] shows a significant change in the main-chain dihedral angles of B24 Phe, allowing B25 Phe to fold outwards from the core of the molecule. If this change were repeated in the complete insulin monomer, the resulting conformational change would be consistent with current ideas of conformational change as a requirement for the expression of biological activity.

The results presented here are consistent with an unfolding of the B-chain from B23 to B30. This change, which involves disruption of the hydrogen bond between B23 carbonyl oxygen and A21 nitrogen and the hydrogen bond between B25 nitrogen and A19 oxygen, is a more radical conformational disturbance than is seen in DPI, where only the B25–A19 hydrogen bond is lost. Although these results do appear to give an indication of the extent to which the C-terminal region of the B-chain may be unfolded in the insulin monomer in solution, it is important to consider them in the light of the experimental conditions. The conditions used range from low pH to near physiological pH and, apart from the marked difference in the effects on DAA insulin, they are consistent within this pH range.The chromatographic process involves both the presence of organic solvent and interaction with the hydrophobic column packing. There is no analogue of these in solution under physiological conditions. It is probable, however, that binding of insulin to its receptor involves a hydrophobic interaction[28], and the behaviour of the B-chain under the hydrophobic experimental conditions may give some indication of its potential for flexibility in a hydrophobic environment *in vivo*.

The predictions for the B23 unfolded model give the best fit to the experimental results for DPI and DOI. The apparent hydrophilicity of DOI tends to be understated, however, and an additional model has been produced to examine the possibility that a further unfolding may take place at B20, as described under Experimental. Predictions based on this model are essentially indistinguishable from the B23 unfolded model, so no theoretical conclusions could be drawn.

The observed difference in the RET for DAA insulin is not predicted by any of the calculations. It has been suggested[17] that the removal of A21 Asn is likely to lead

to a disruption of the conformation of insulin by placing a negative charge close to the hydrophobic core of the molecule. In an earlier HPLC investigation of insulin analogues[11] we found, unexpectedly, that DAA insulin eluted before native bovine insulin in the shallow gradient system used. This finding was explained by the fact that, at pH 2, the carboxylate of A20 Cys is likely to be partially protonated, leading to a reduction in charge. In the current series of experiments, DAA elutes slightly after native insulin at pH 2, but still close to the position predicted by all methods. At pH 7, however, its RET is significantly greater than both the pH 2 results and all of the predictions for pH 7.

This result suggests that there is a significant conformational disturbance when the carboxylate of A20 becomes charged. Looking at the structure of the molecule, the residues closest to A20 are B24 and B25, the A20–B24 C-α to C-α distance being 4.17 Å and the A20–B25 distance being 6.61 Å in the crystal structure for molecule 1. If we assume, however, that the B-chain is unfolded from B23, the accesssibilities of B24 and B25 will be significantly increased so that any further increase due to the repulsive effect of the charge on B20 is likely to be small. Predictions at both pH 2 and 7 for B23 unfolded insulin, where the accessibilities of both B24 and B25 are maximized, give little indication of an RET for DAA insulin as large as the experimental value of 2.0 at pH 6.5, whilst the predicted result for B23 and B24 unfolded DAA is similar at both pH values. These results suggest a more drastic disturbance in the conformation of DAA insulin when the terminal carboxylate of A20 is charged; possibly the altered charge environment in the B19–B20 region acts to destabilize the conformation of the molecule as a whole, leading to great exposure of core residues.

CONCLUSION

It is clear that the approach adopted here has some value in interpreting the HPLC elution of closely related molecules. Regnier[26] proposed that the interaction of a folded protein with the stationary phase in a chromatographic system is dependent on a chromatographic contact area which, in reversed-phase chromatography, may occupy a significant proportion of the surface of the molecule. From our previous work[11], we have concluded that, with a small molecule such as insulin, the reversed-phase chromatographic contact area is effectively the whole surface of the molecule and that a change at any position on the surface is likely to affect the chromatographic behaviour. It has been possible, using this assumption, to combine experimentally derived HPLC elution coefficients with accessibility calculations in interpreting the elution behaviour of insulin analogues and to provide experimental evidence for the extent of flexibility of the C-terminal portion of the insulin B-chain.

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