Journal of Chromatography, 285 (1984) 319–331
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,400

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF INSULIN

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(Received October 26th, 1983)

SUMMARY

The elution behaviour of a selection of sequence variant and modified insulins has been compared on a C_{18} reversed-phase column. Observed elution times were compared with those expected from the nature of the differences from bovine insulin. In some cases prediction rules established for peptides are adequate to explain the observed elution and in others detailed knowledge of the structure of the protein is of considerable importance in understanding elution behaviour.

INTRODUCTION

Considerable progress has been made in the use of reversed-phase chromatography in the separation of small peptides and, more recently, several reports of separations of larger polypeptides and proteins have appeared[1–3]. The technique becomes particularly important in the final stages of protein purification where products of rather similar size and charge properties may remain after more conventional separation procedures. To understand the limitations of this method there are major advantages in studying the chromatographic behaviour of a protein whose physico-chemical properties are well understood. In particular, a known three-dimensional structure provides a basis from which one might predict the nature of surfaces available to interact with the reversed-phase support and solvent in different experimental conditions.

Several recently published separations of polypeptide mixtures using reversed-phase liquid chromatography suggest that, where the peptide is small enough for there to be little stable secondary or tertiary structure in solution under the conditions of the experiment, the elution position of a peptide molecule appears to be determined by the sum of the hydrophobicities of its constituent amino acids[4,5]. O'Hare and Nice[4] have found it possible to predict with reasonable accuracy the elution behaviour of peptides containing up to fifteen residues by summing the fragmental hydrophobic constants, as determined in an octanol–water system by Rekker[6], of the five most hydrophobic residues. Meek[5] has proposed that retention coefficients, determined from experiments on the elution behaviour of polypeptides on a reversed-phase column, calculated for all amino acids will be of greater predictive value and will be applicable to peptides of up to twenty residues. These coefficients have been

0021-9673/84/$03.00

used to predict successfully the elution order of the peptides in O'Hare and Nice's series.

The application of these methods to larger peptides and proteins which may be more closely related to one another than those used in the above studies presents several problems. First, molecules which retain some secondary and tertiary structure will not necessarily elute in the position predicted by methods which give equal weightings to the contributions of all of the residues. It is to be expected that residues on the surface of the molecule will have a greater effect on elution behaviour than those which are not exposed to the solvent. Secondly, some proteins have been found to bind irreversibly to the stationary phase[4] and, thirdly, the methods used for small peptides use gradient elution[4,5] which cannot be relied upon to separate closely related peptides or proteins of the sort used in the present study.

In the work reported in this paper we have examined the chromatographic behaviour of several naturally occurring insulins and proinsulins and a number of beef insulin analogues. The use of analogues allows the effects of the addition of a range of chemical groups to a limited number of sites to be evaluated while the use of a variety of naturally occurring insulins allows us to study the effects on elution of alteration of the amino acid residues present at a wider variety of sites.

Several studies on the analysis of insulins on reversed-phase liquid chromatography have been described in the literature using a variety of organic modifiers, buffers and "ion pairing" agents to optimise column performance[7–12]. We have endeavoured to use a limited range of conditions in order that comparative elution behaviour might reflect the properties of the molecules chromatographed. In the present study an acetonitrile/phosphate based system is used to investigate the elution behaviour of beef, human, pork, mouse 1 and 2, turkey and the hystricomorph rodent cuis insulins, beef proinsulin and a series of beef insulin analogues. Separations were either isocratic or used very shallow gradients; flow programming was also used.

MATERIALS AND METHODS

High-performance liquid chromatography (HPLC)

This was carried out using a Varian 5000 liquid chromatograph equipped with an ODS Ultrasphere column (250 × 4.6 mm) supplied by Altex. Flow gradients were used in the separation of the beef insulin analogue mixture, but all other experiments were carried out at a constant flow-rate of 1 ml/min. Details of the flow gradients are given in the Results and Discussion section. The mobile phase consisted of two components. Solution A contained 0.1 *M* phosphate buffer, made up by adjusting the pH of a 0.1 *M* aqueous solution of sodium dihydrogen phosphate with orthophosphoric acid. Batches of this solution were made up to pH 2, 2.5, 3 and 3.5. For some experiments, 10 m*M* perchloric acid was included. Details of the use of these various solutions are given in the Results and Discussion section. Solution B consisted of acetonitrile. All reagents used for solution A were AnalaR grade, supplied by BDH; water was obtained from the laboratory still. Acetonitrile, HPLC grade, was supplied by the Rathburn Chemical Company. The Varian chromatograph was programmed to operate isocratically with solution B percentages of 27, 28, 29 and 30 or to run shallow gradients between 27 and 31%. Further details of the use of these gradients and percentages are given in the Results and Discussion section.

Materials

Pork insulin was supplied by Novo, human and beef insulins and proinsulin by Eli Lilly. Turkey insulin, mouse insulins 1 and 2, cuis insulin and DAA (des Ala B30, des Asn A21 bovine insulin) were prepared in this laboratory. Small residual amounts of beef insulin analogues which were initially prepared for a programme of investigation of the structure and function of insulin by D. Brandenburg, H. J. Friesen, D. G. Lindsay and R. A. Pullen (see refs. 13 and 14) were used. They included A0 Arg-Arg, A0 Arg, A1 succinyl, A0 Glu, A0 L-Phe, A0 D-Phe, A1 acetyl and A1 thiazolidine analogues of beef insulin.

RESULTS AND DISCUSSION

Before describing the experimental results in detail, it is appropriate to consider the expected effects of the experimental conditions on the conformation of the insulin molecule. In its most intensively studied crystalline form, porcine insulin exists as a hexamer. The crystal asymmetric unit is a dimer, the two monomers exhibiting minor differences in the arrangement of their polypeptide chains. The monomer is a globular protein having two polypeptide chains, designated A and B, linked by disulphide bonds. It has a hydrophobic core, two predominantly non-polar surfaces and one large polar surface (see Fig. 1). Of the non-polar surfaces, that involving B23–B26, B12 and B16 is involved both in dimer formation and in receptor binding while that involving A13 and 14, B14, B17 and B18 is involved in inter-dimer contacts in the hexamer. In solution, various aggregation states can exist; monomer, dimer or hexamer. In the present series of experiments, all samples were made up in phosphate buffer pH 2 at 1 mg/ml. Under these conditions, insulin exists in a monomer–dimer equilibrium[15]. During the chromatographic process itself, the presence of acetonitrile would be expected to interfere with any hydrophobic interaction between monomers. Chromatographic runs carried out in the pH range 2.7–3.5 where dimer formation is more favoured[16] are not dramatically different from those carried out below pH 2.7, suggesting that the solvent is sufficient to suppress dimerisation. Considering the effects of the experimental conditions on the conformation of the monomer, any deviations from native conformation would be expected to be due to either the low pH or to the presence of organic solvent. Circular dichroism spectroscopy suggests that there is little change in conformation between pH 8 and pH 2[17], so we may expect that the relatively low pHs used in the present study will not produce large deviations from native conformation. Circular dichroism spectra recorded in the presence of organic solvent show several changes[18]. These have been attributed to changes in the environment of tyrosine residues on dissociation of the dimer by the solvent, some minor conformational alterations and an increase in the α helix content. A possible site for the induction of additional helix is in the B1–B8 region which is displaced to form an extension of the B chain helix in some crystal forms[19,20]. We may expect some structural changes in our system, but these should represent relatively minor variations in native conformation.

Beef insulin analogues

A series of experiments was carried out with the beef insulin analogues listed in the Materials and Methods section. Initially, each of these was run in isocratic

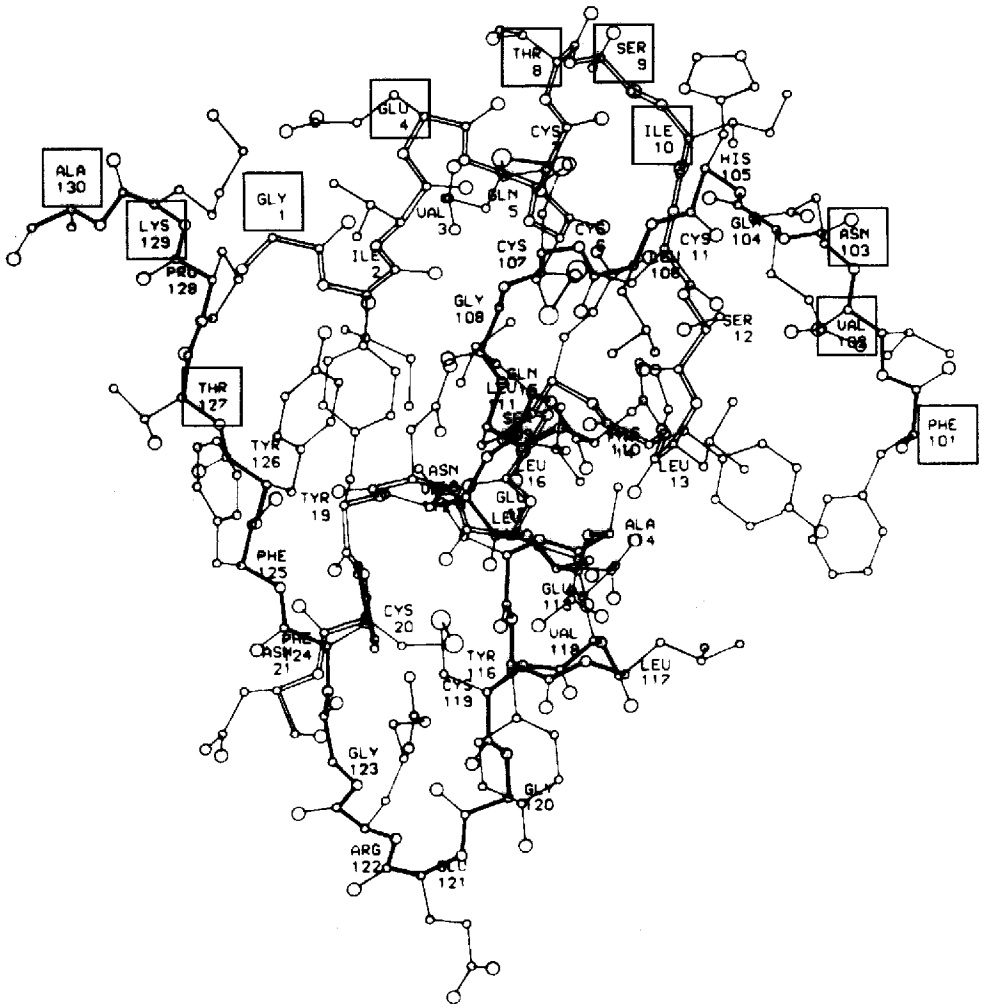

Fig. 1. View of the pork insulin monomer showing the residues involved in substitution or derivatisation in the various insulins and insulin analogues[15].

conditions to determine the ideal conditions for its elution and its relative elution time with respect to beef insulin. These runs were carried out at acetonitrile concentrations of 27, 28, 29 and 30%. On the basis of these preliminary trials, several gradient systems were tried in an attempt to elute all of the analogues in a single run. A near complete separation was achieved with only A1 acetyl and A0 Glu coeluting (see Fig. 2). Preliminary trials showed that A0 L-Phe and A0 D-Phe insulins behaved identically and so A0 D-Phe insulin was not used subsequently.

In the preliminary runs, perchloric acid was not included in solution A, but in the gradient runs 10 m*M* perchloric acid was included. The details of the system used for the final separation are as follows: acetonitrile concentration was at 28% for 95 min then increased to 29%; a further increase to 30% took place at 115 min. The following flow gradient was used concurrently: over the first 25 min there was a linear

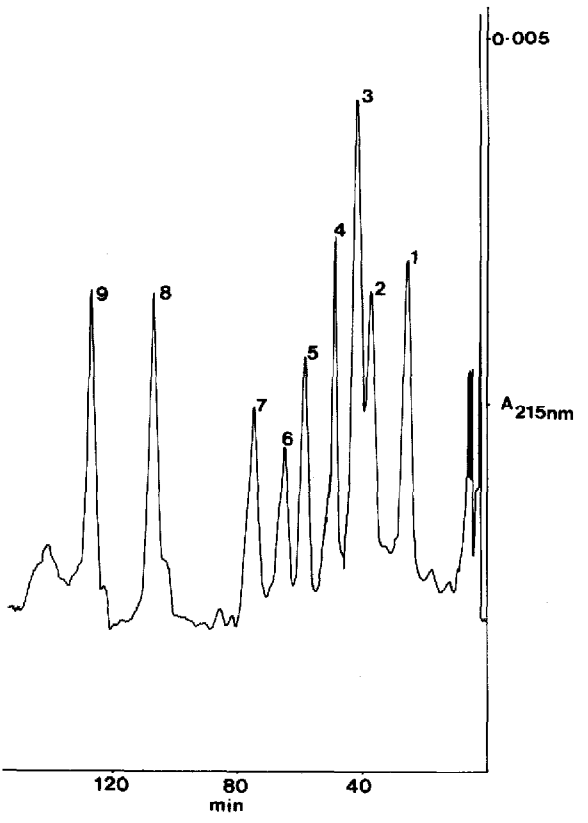

Fig. 2. Chromatographic separation of beef insulin analogues. Peaks: 1 = A0 Arg-Arg; 2 = A0 Arg; 3 = DAA; 4 = beef insulin; 5 = A1 succinyl; 6 = A1 acetyl/A0 Glu; 7 = A0 L-Phe; 8 = B1 acetyl; 9 = A1 thiazolidine insulin. See text for details of gradient elution and flow program.

decrease in flow from 1 ml/min to 0.5 ml/min; flow was held at 0.5 ml/min for 15 min and then increased linearly to 1 ml/min over 10 min and this flow-rate was held for the remainder of the run. The final elution order was as follows: 1, A0 Arg-Arg; 2, A0 Arg; 3, DAA beef; 4, beef (unmodified); 5, A1 succinyl; 6, A1 acetyl/A0 Glu; 7, A0 L-Phe; 8, B1 acetyl; 9, A1 thiazolidine.

A number of general points arise from these results. First, it was not possible to elute all of the analogues under isocratic conditions, indicating that the differences between the molecules as seen by the chromatographic system were greater than between the naturally occurring insulins used which all eluted isocratically at 28%. Secondly, under the gradient conditions used, the relative elution times to beef insulin tend to have a wider range than those for most of the naturally occurring insulins. Thirdly, the elution order can be explained in terms of the character of the groups added or deleted and the known structure of the insulin molecule.

In considering the addition or deletion of chemical groups, these can be considered as belonging to two categories, those involving the addition of an uncharged group to the N terminus of one of the chains and those which involve the additions of one or more amino acids to the N terminus of the A chain or the deletion of an

amino acid from the C terminus of the A chain. In the first case the number of positive charges is reduced by one and, in the second case, it either remains the same or is increased. Of the analogues used, A1 acetyl, B1 acetyl and A1 thiazolidine insulins all have one less positive charge which would normally be found on the protonated amino group at the N terminus of the chain to which the substituents are attached. The succinyl group can also be considered as an uncharged group since its free carboxylate should be protonated at pH 2.

All of the groups which cause a reduction in the number of positive charges result in the analogue eluting after beef insulin. This is consistent with an increase in the overall hydrophobicity of the molecule. Unfortunately it is not possible to consider their elution behaviour in terms of Meek's retention coefficients[5] since, of the groups used, only acetyl is included in his table. It is possible, however, to interpret these results on the basis of a qualitative assessment of the possible effects on elution behaviour of the modifications present in the various analogues. Considering the A1 analogues first, A1 succinyl elutes closest to beef insulin. Although the free carboxylate is protonated at pH 2, there still exists the possibility for hydrogen bonding to water molecules which significantly reduces the effects of the presence of two methylene groups and the removal of the positive charge on the A1 glycine; both of these changes would, by themselves, tend to increase the overall hydrophobicity of the molecule and would be expected to prolong the retention time. A1 acetyl elutes after A1 succinyl. Acetyl has a single methyl group as against the two methylene groups of succinyl and a single oxygen atom on the carboxyl involved in the amide bond with the A1 glycine. The increase in retention time as compared to A1 succinyl is most probably due to the lack of a free carboxylate group capable of participating in hydrogen bonding. The possibility that the oxygen of A1 acetyl may not be fully exposed to the solvent will be discussed when considering the effect of B1 acetyl elution. A1 thiazolidine elutes much later than the other A1 analogues. This appears to be due simply to the presence of a large hydrophobic group coupled to the removal of the charge on the A1 glycine. X-ray diffraction studies of crystals of A1 thiazolidine insulin have shown that the thiazolidine group extends beyond the surface of the monomer, associated with a distortion of the A chain helix A2–A5[13]. In solution, this would lead to the thiazolidine group being completely exposed to the solvent. Circular dichroism studies suggest that there is no significant disruption of conformation in solution although the tendency to form dimers is less than with native beef insulin[13]. These observations suggest that the greater overall hydrophobicity of A1 thiazolidine as compared to native beef insulin is simply due to the presence of the added group and not to any conformational disturbance leading to exposure of the hydrophobic core of the molecule.

B1 acetyl elutes considerably later than A1 acetyl insulin, indicating that the elution behaviour of these analogues is dependent on more than the quantitative addition of an acetyl group to the molecule. In the native conformation of beef insulin the environments of the N termini of the A and B chains are very different, the B1 terminus being at the end of a section of extended polypeptide chain which may be relatively unconstrained in solution while the A1 terminus is situated in a pocket on the surface of the monomer, close to the region involved in inter-monomer contacts in the dimer. It is possible, then, that the acetyl group of A1 acetyl insulin is not fully exposed to the solvent while that of B1 acetyl is; the difference in elution time of B1

acetyl from beef insulin would then represent the full effect of the addition of an acetyl group with the loss of one positive charge. In the initial series of isocratic runs it was not possible to elute either B1 acetyl or A1 thiazolidine insulins in the conditions which were found to be ideal for native beef insulin. It has already been noted that the conformation of A1 thiazolidine insulin is altered to allow full exposure of the thiazolidine group, thus exposing the substituted A1 terminus to the degree expected in B1 substituted insulins. The elution behaviour of the A1 acetyl analogue suggests that there is less conformational disturbance and that the acetyl group is less exposed to the solvent.

All of the analogues with added amino acids are considered as A0 analogues. The total number of charged groups is altered only where amino acids with charged side chains are added. This means in practice that, of the analogues used, only those with added arginyl residues will show a change in charge since the free carboxylate of A0 Glu will be protonated at pH 2. It may be predicted, then, that both A0 Glu and A0 Phe will elute after beef insulin and that is what happens in practice. It is interesting to compare the elution of A0 Glu with A1 succinyl insulin since both of these analogues have a carboxylate group attached to two methylene groups projecting beyond the surface of the molecule. The later elution of A0 Glu insulin may be interpreted to mean that more of this hydrocarbon chain is exposed to the solvent by virtue of the fact that it is separated from the A1 glycine by one additional carbon atom in this analogue, thus causing an increase in overall hydrophobicity which more than offsets the effect of the retention of a protonated amino terminus. In Meek's system[5], glutamate is given a negative retention coefficient at pH 2.1 which would predict elution before native beef insulin; this is not consistent with the results obtained here and perhaps indicates the limitation of applicability of retention coefficients to molecules where three-dimensional structure is preserved under the chromatographic conditions. A0 Phe elutes significantly after A0 Glu. This is consistent with the presence of the aromatic ring which would be expected to make a significantly greater contribution to overall hydrophobicity.

The two analogues which have amino acids added with positively charged side chains both elute before beef insulin. This is in accord with predictions made both on the basis of the above arguments and with Meek's retention coefficients. It is worthy of note, however, that the retention coefficient for arginine is less negative than that for glutamic acid. In the gradient system used to elute all of the analogues together, the relative elution time of A0 Arg-Arg to A0 Arg insulin is similar to that of A0 Arg to beef insulin. It is difficult to make any quantitative judgement on this since a flow gradient was used at this point. In the preliminary series of isocratic runs, however, the difference in elution times of A0 Arg and beef insulins was smaller than that of A0 Arg-Arg and A0 Arg insulin. This is consistent with one of the added arginyl residues being less exposed to the solvent due to conformational effects.

The other analogue which eluted before beef was DAA beef insulin. This has the A21 Asn deleted, causing considerable disruption to the conformation at neutral pH by placing a negative charge on A20 Cys which tends to cause repulsion of adjacent hydrophobic residues. The finding that DAA beef insulin elutes closer to native beef insulin than any of the analogues which have added amino acids might suggest, therefore, that conformation is not a crucial factor in determining elution behaviour. This would be in conflict with the other HPLC evidence and the theo-

retical arguments presented elsewhere in this paper. A possible explanation of this discrepancy is that the carboxylate group of A20 Cys is partially protonated at pH 2. This would reduce the tendency to disruption created by the repulsion of hydrophobic groups and allow the molecule to attain a conformation similar to that of the native beef insulin monomer in solution.

One factor in favour of this hypothesis is the elution of DAA before beef. If the conformation was significantly disturbed and the hydrophobic core of the molecule exposed to the solvent, DAA would elute after native insulin.

Naturally occurring insulins

The general elution behaviour of these insulins was investigated and a series of experiments was carried out with beef, human and pork insulins at a range of pHs. All chromatographic runs were carried out isocratically with an acetonitrile content of 28%. Solution A consisted of 0.1 *M* phosphate buffer only except where conditions were optimised to produce maximum resolution in the separation of a beef–human–pork insulin mixture. In this case 10 m*M* perchloric acid was included as described in Materials and Methods. The inclusion of chaotropic salts in the mobile phase has been found to give increased peak sharpness and to increase resolution[4,5,7]. Their mechanism of action is unclear. We have found that the inclusion of perchlorate gives a significant increase in resolution with a considerable increase in retention time. For small peptides it has been suggested that chaotropic agents interfere with folding and ensure that the whole peptide is seen by the bonded phase. This is a less likely explanation for a folded protein. Possibly these agents aid in the dispersal of C_{18} chains on the matrix surface, permitting more intimate contact with the mobile phase and its solutes. An acetonitrile concentration of 28% was chosen as optimal for the insulins used. Small changes in the concentration of solvent can have large effects on retention of the sample[4] and we have found that a change of 1% either seriously degraded the resolution or gave unacceptably long retention times.

The best separation achieved of a beef–human–pork insulin mixture was carried out at pH 2 with 10 m*M* perchlorate in solution A (Fig. 3). Complete resolution of human and pork insulins was achieved, the elution order being beef followed by human followed by pork. The sequence differences between these insulins are given in Table I. It will be seen that beef and pork insulins differ in the residues at A8 and A10 and that pork and human insulins differ only at B30. A8 and A10 are situated on the surface both of the monomer and the dimer, in the loop of the A chain enclosed by the intrachain disulphide bond between A6 and A11. The A8 residue is directly adjacent to A7 Cys which participates in an interchain disulphide bond. It is to be expected that the structure of this region would be preserved, under the conditions of the experiment, by the disulphide bonds. The B30 residue is situated close to the hydrophobic surface involved in dimer formation although it is quite exposed to the solvent in all aggregation states.

Considering the elution order in terms of Meek's retention coefficients, it is convenient to examine only those residues which differ in the three insulins, allowing a qualitative prediction to be made of elution order. The predicted insulin elution order is beef, pork, human which is different from that observed both in the present and other work[7–12]. It is noteworthy that Terabe *et al.*[8] chromatographed the B30

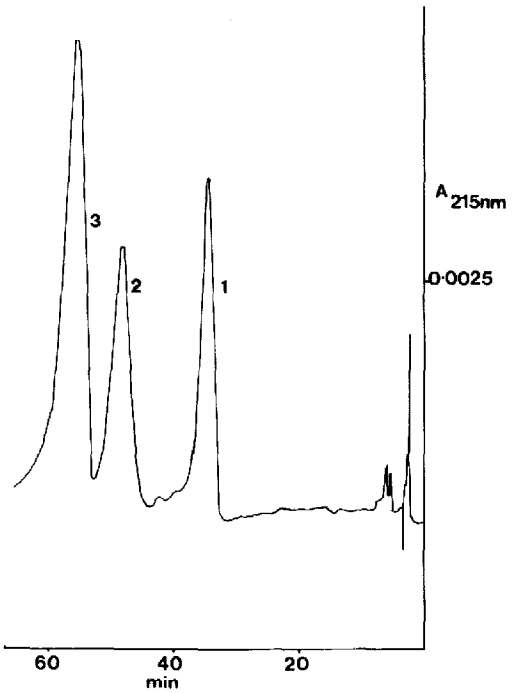

Fig. 3. Chromatogram of beef (1), human (2) and pork (3) insulins. pH 2 phosphate buffer with 10 m*M* perchloric acid.

Thr analogue of beef insulin which eluted before unmodified beef insulin. It would appear that, in the conditions of the present study, Thr makes the molecule more hydrophilic, possibly by forming hydrogen bonds with water molecules. It is possible that, under other conditions, it could be involved in adsorption phenomena with the column packing.

A series of runs with beef–human–pork insulin mixtures was carried out at pHs between 2 and 3.5 using 0.1 *M* phosphate buffer as solution A. Resolutions were calculated from the formula

TABLE I

SEQUENCE DIFFERENCES IN NATURALLY OCCURRING INSULINS AND THEIR RELATIVE ELUTION TIMES (R.E.T.) TO BEEF INSULIN WHEN CHROMATOGRAPHED UNDER ISOCRATIC CONDITIONS AT 28% ACETONITRILE IN pH 2 PHOSPHATE BUFFER

	A4	*A8*	*A9*	*A10*	*B1*	*B2*	*B3*	*B9*	*B27*	*B29*	*B30*	*R.E.T.*
Beef	Glu	Ala	Ser	Val	Phe	Val	Asn	Ser	Thr	Lys	Ala	1
Human		Thr		Ile							Thr	1.31
Pork		Thr		Ile								1.45
Mouse 1	Asp	Thr		Ile			Lys	Pro			Ser	1.32
Mouse 2	Asp	Thr		Ile			Lys			Met	Ser	2.57
Turkey		His	Asn	Pro	Ala	Ala			Ser			0.38

$$R = \frac{2\Delta t}{W_1 + W_2}$$

where Δt is the difference in retention times and W_1 and W_2 are the widths of the two peaks. The ratios of the elution times of the components of the sample mixtures are presented as relative elution times. The data are given in Table II. It will be noted that there is a steady decrease in resolution with increasing pH while the relative elution time remains constant. The observation that resolution is improved at low pH has been made by various workers[4,21]. This is explained by possible reduction of adsorptive interactions with the column packing or by suppression of ionisation of acidic groups on the protein molecules. In addition to the two C terminal groups which would be expected to ionise in the pH range investigated, insulin has four Glu residues which would be expected to exist increasingly in the ionic form towards the higher end of the pH range investigated. Both of the postulated mechanisms would therefore be expected to operate. Since there is no substitution of any of these acidic residues, all three insulins are affected equally.

Mouse insulins 1 and 2 and turkey insulin were chromatographed at 28% acetonitrile in pH 2 phosphate buffer without perchlorate; their relative elution times to beef insulin and the differences in sequence between these insulins and beef are given in Table I. Mouse insulin is of particular interest since it shows a polymorphism which is easily resolved by the chromatographic system. Both mouse insulins were retained for longer than beef insulin with mouse 1 eluting before mouse 2. Meek's retention coefficients predict that both mouse insulins will elute after beef insulin but give a reversed elution order for mouse 1 and 2. The mouse 1 sequence has two lysine residues which would be positively charged in our chromatographic system, thus rendering the molecule less hydrophobic than mouse 2 which has a single lysine. This can be considered as being roughly equivalent to the single lysine of beef insulin which, although it is in a different position in the B chain, is equally exposed to the solvent and can be expected to have a similar effect on elution behaviour. The general effect of a positively charged group causing earlier elution is similar to that observed with the A0 Arg and A0 Arg-Arg beef insulin analogues. It is noted that the sequence differences from beef insulin all affect residues situated on the surface of the monomer. None of the residues on the mainly hydrophobic surfaces is affected.

The sequence differences between turkey and beef insulins also affect surface

TABLE II

RESOLUTIONS AND RELATIVE ELUTION TIMES OF BEEF–HUMAN AND HUMAN–PORK INSULIN PAIRS AT VARIOUS pHs SHOWING THE DETERIORATION IN RESOLUTION WITH INCREASING pH WHILE RELATIVE ELUTION TIME REMAINS RELATIVELY CONSTANT

pH	*Beef–human*		*Human–pork*	
	R.E.T.	*Resolution*	*R.E.T.*	*Resolution*
2	0.76	1.99	0.9	0.69
2.5	0.74	1.8	0.89	0.73
3	0.78	1.05	0.91	0.46
3.5	0.79	0.974	0.91	0.4

residues. The overall effect is to make the molecule less hydrophobic, mainly as a result of the substitutions at B1 and B2. Although a histidine is present at A8 which has a positively charged imidazole side chain, the effect of this amino acid on overall hydrophobicity appears to be relatively neutral, both in terms of Meek's retention coefficients[5] and of the hydrophobicity figures given by Nozaki and Tanford[22]. The effect of the positive charge appears to be counteracted by the hydrophobicity of the imidazole ring itself. The relative elution time of turkey to beef insulin is given in Table I. It will be seen that it elutes before beef insulin. This is in agreement with a qualitative prediction based on Meek's retention coefficients.

Cuis insulin has recently been sequenced[23] and shows extensive differences from beef insulin. These differences appear to involve the surface of the molecule, including the areas involved in inter-monomer and -dimer contacts in beef insulin. The most notable feature of this sequence is the presence of a greater number of amino acids with positively charged side chains than beef insulin. This suggests that the molecule should be less hydrophobic than beef insulin and this may also be qualitatively predicted by Meek's retention coefficients. Cuis insulin was chromatographed at 28% acetonitrile in pH 2.5 phosphate without perchlorate and eluted well before beef in accordance with predictions based on sequence data. Rather similar early elution times relative to beef insulin have been observed for other insulins from hystricomorph rodents such as the guinea pig and coypu reflecting the production by these animals of more hydrophilic insulins in the absence of an ability to aggregate[25].

Beef proinsulins

Proinsulin is the precursor for insulin and is characterised by a region of polypeptide chain, of about 30 residues, connecting the B30 to the A1 residues of insulin[24]. It has been suggested that the kinetics of the cleavage process implies that these residues are situated on the surface of the molecule[15]. The detailed conformation of the C peptide is not yet known but is presently under investigation[26]. It

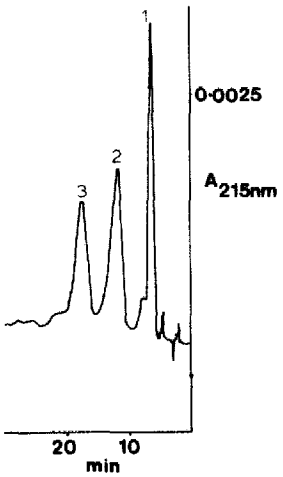

Fig. 4. Chromatogram of beef insulin (1), beef proinsulin (C48 Pro) (2) and beef proinsulin (C48 Leu) (3). See text for details of gradient elution.

is generally agreed, however, that the C peptide is probably situated on the face of the molecule not involved in aggregation. This is supported by the fact that proinsulin may self associate and by antibody binding studies[26,27].

It has been shown that in cattle there are two proinsulins, differing in one residue only, C48 Pro being substituted by Leu, which may be separated on reversed-phase HPLC[28]. A mixture of both of these proinsulins and beef insulin was chromatographed (Fig. 4). It was necessary to use a gradient of 30 to 34% over 30 min to elute this mixture satisfactorily. The presence of perchlorate in solution A tended to degrade the resolution of the proinsulins, apparently by a direct effect on the protein itself. The mixture was eluted, therefore, with no perchlorate in solution A. It is difficult to make a direct comparison between this result and the results for the insulins and insulin analogues since a rather steeper gradient was used. Several comments may, however, be made. First, the elution order was beef insulin followed by (C48 Pro) proinsulin followed by (C48 Leu) proinsulin. Secondly, the difference in the relative elution times to beef insulin of the two proinsulins is very large. This is slightly surprising since proinsulin is regarded, at least at neutral pH, as being generally more hydrophilic than insulin, making it more suitable as an intracellular transport form[29]. Complete resolution of these materials was achieved with little difficulty in the gradient system used and the difference in their elution behaviour was considerably greater than with human and pork insulins. It is possible that there is a conformational difference between these proinsulins which is responsible for the difference in their behaviour.

CONCLUSIONS

It has been shown that, in carefully controlled conditions, reversed-phase chromatography can separate mixtures of proteins which have minimal differences in structure. Separations are possible between insulins which differ by a single uncharged residue in a position where it is unlikely to have a significant effect on overall conformation, as evidenced by the human–pork insulin separation in this and other studies[8,30]. This sensitivity in the separation of closely related proteins allows the method to be used to detect naturally occurring polymorphic proteins where more than one gene product is present. The bovine proinsulins used in this study differ by one amino acid in 81 and a separation of three mutant human insulins, including a B25 analogue, has recently been published[30].

In many cases it seems that the partition coefficient for different insulins is determined by the entire exposed surface of the molecule rather than any preferential involvement of a particular surface region. The observation that a particular modifying group, such as acetyl, has a different effect on elution behaviour depending on its site of attachment to the molecule may be taken as a reflection of its degree of exposure on the surface, as determined by the overall three-dimensional structure. The coelution of the A0 L-Phe and A0 D-Phe analogues suggests that detailed stereochemistry is important only in so far as it influences the exposure of hydrophobic or hydrophilic groups on the surface of the molecule.

Predictive systems based on amino acid composition for the elution order of polypeptides must be used with some caution when applied to peptides which retain some three-dimensional structure in the chromatographic conditions since the effect

of any residue on elution will be influenced by its degree of exposure to the solvent. Predictions appear more reliable where a larger number of amino acid differences exist between two molecules.

ACKNOWLEDGEMENTS

We wish to thank all those who made materials available including D. Brandenburg, H. J. Freisen, M. Bajaj, J. Pitts and B. Frank and the Science and Engineering Research Council for financial support.

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