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Chemical reactivity of the functional groups of insulin

Concentration-dependence studies

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A modification to the competitive labelling procedure of Duggleby and Kaplan [(1975) *Biochemistry* **14**, 5168–5175] was used to study the reactivity of the *N*-termini, lysine, histidine and tyrosine groups of insulin over the concentration range 1×10^{-3}–1×10^{-7} M. Reactions were carried out with acetic anhydride and 1-fluoro-2,4-dinitrobenzene in 0.1 M-KCl at 37°C using Pyrex glass, Tefzel and polystyrene reaction vessels. At high concentrations all groups had either normal or enhanced reactivity but at high dilution the reactivities of all functional groups became negligible. This behaviour is attributed to the adsorption of insulin to the reaction vessels. The histidine residues show a large decrease in reactivity in all reaction vessels in the concentration range 1×10^{-3}–1×10^{-5} M where there are no adsorption effects and where the reactivities of all other functional groups are independent of concentration. With polystyrene, where adsorption effects become significant only below 1×10^{-6} M, the reactivity of the phenylalanine *N*-terminus also shows a decrease in reactivity between 1×10^{-5} and 1×10^{-6} M. In 1 M-KCl insulin does not adsorb to Pyrex glass and under these conditions the histidine reactivity is concentration-dependent from 1×10^{-3} to 5×10^{-6} M and the B1 phenylalanine α-amino and the B29 lysine ε-amino reactivities from 5×10^{-6} to 1×10^{-7} M, whereas the reactivities of all other groups are constant. These alterations in reactivity on dilution are attributed to disruption of dimer–dimer interactions for histidine and to monomer–monomer interactions for the phenylalanine and lysine amino groups. It is concluded that the monomeric unit of insulin has essentially the same conformation in its free and associated states.

The structure of insulin in its associated states has been extensively studied by X-ray crystallography (Chothia *et al.*, 1983; Blundell, 1979; Blundell *et al.*, 1971). At sufficiently low concentrations, insulin dissociates into the free monomeric unit which, on the basis of current evidence, is the physiologically active species (Blundell, 1979; Holladay *et al.*, 1977; Milthorpe *et al.*, 1977). It is therefore essential to determine if there are differences in secondary and tertiary structure between the monomeric unit in its free form and in its associated states. At present, there is conflicting evidence in the literature on this point based on c.d. studies. Wood *et al.* (1975) have concluded that the conformation of the monomeric unit remains essentially unaltered on dissociation, whereas Pocker & Biswas (1980, 1981) claim that there are substantial changes.

Studies on the free insulin monomer are difficult due to the lower concentrations of this protein that must be employed. In this paper, we describe an improvement to the competitive labelling technique (Duggleby & Kaplan, 1975; Kaplan *et al.*, 1971) which increases its sensitivity to the point where the reactive properties of individual functional groups in proteins and, by inference, conformation may be studied at high dilutions. This approach made possible the study of insulin at concentrations where the free monomeric species predominates.

Experimental

Rationale

The competitive labelling method (Kaplan *et al.*, 1971) as modified by Duggleby & Kaplan (1975)

Abbreviations used: Dnp-F, 1-fluoro-2,4-dinitrobenzene; Dnp-, 2,4-dinitrophenyl-.

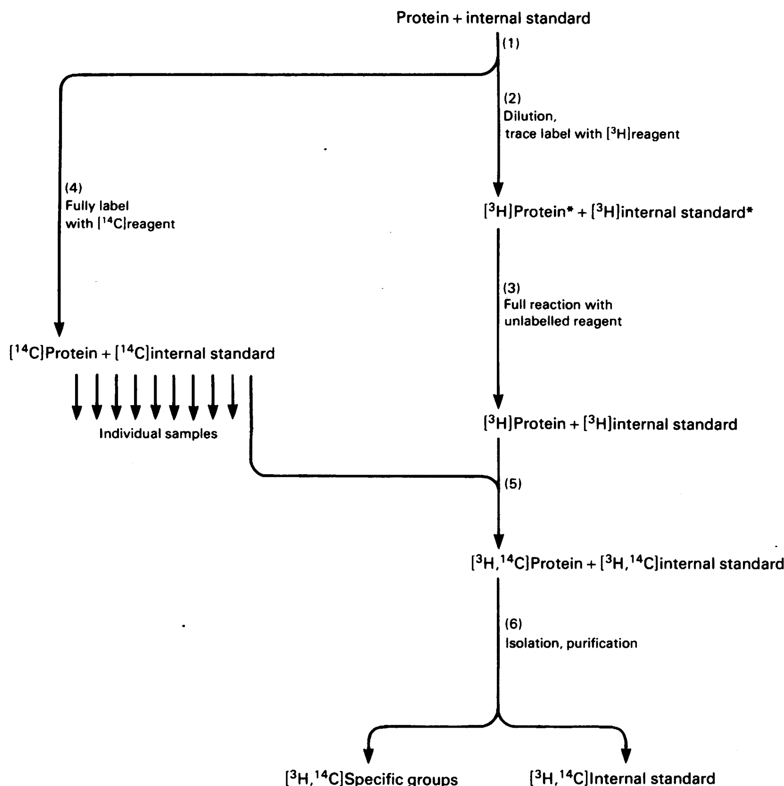

Fig. 1. *Flow diagram of experimental procedure*

consists of four main steps. These are: (i) treatment of a mixture of the protein under study and a suitable standard nucleophile with a trace of a ^{3}H-labelled electrophilic reagent; (ii) treatment of the mixture with ^{14}C-labelled reagent under denaturing conditions to render the protein and internal standard chemically homogeneous; (iii) separation, purification and quantification of the internal standard; and (iv) partial or total hydrolysis of the protein, followed by purification and quantification of a peptide or amino acid containing the functional group under study. In the present procedure, a modification, based on a competitive labelling procedure reported by Kraal & Hartley (1978), has been introduced in order to carry out reactivity studies at low concentrations of protein. From experience, as the amount of protein is decreased, the limiting factor in obtaining accurate quantification of the ^{3}H/^{14}C ratio is the amount of ^{14}C-labelled reagent incorporated in step (ii). To overcome this difficulty, a stock solution of protein and internal standard is prepared. This is then divided [Fig. 1, (1)] into two aliquots. One aliquot is diluted to the appropriate concentration and step (i) carried out as before [Fig. 1, (2)]. In step (ii), full reaction is now carried out with non-radioactive reagent to achieve chemical homogeneity [Fig. 1, (3)]. The other aliquot is fully reacted with ^{14}C-labelled reagent [Fig. 1, (4)] and equal aliquots of this are added to each trace-labelled sample [Fig. 1, (5)]. Steps (iii) and (iv) remain unchanged.

In this modified procedure, sufficient ^{14}C radioactivity is assured by labelling an appropriate amount of the protein and internal standard separately from the ^{3}H trace-labelled sample. Because the ^{14}C-labelled sample and the experimental sample which has been diluted to the appropriate concentration before trace-labelling are derived from the same stock solution, they must have the same protein-to-internal-standard ratio. It is important to note that the rates being measured for groups in the protein are relative rates, i.e. relative to the internal standard. Therefore, combining the two samples achieves the same result as a direct ^{14}C labelling of the ^{3}H trace-labelled samples and the calculation of reactivities can be obtained from the following equation:

$$\alpha_x r = \alpha_s \frac{(^{3}H_x/^{14}C_x)}{(^{3}H_s/^{14}C_s)} \quad (1)$$

where α_x is the degree of ionizaton of the functional group being studied, α_s the degree of ionization of the internal standard [phenylalanine $pK_a = 9.01$ (Sheffer & Kaplan, 1979); L-β-imidazole-lactate $pK_a = 7.27$ (Cruickshank & Kaplan, 1975)], r the pH-independent second-order velocity constant for the reaction of the functional group relative to that of its internal standard (i.e. phenylalanine for acetylation: imidazole-lactate for dinitrophenylation) and ($^3H_x/^{14}C_x$) and ($^3H_s/^{14}C_s$) the corresponding $^3H/^{14}C$ radioactivity ratios for the groups and internal standard, respectively.

Materials

Pig zinc insulin (0.35% zinc by wt.) was obtained from Connaught Laboratories (Toronto, Ontario, Canada). Amersham Corporation (Oakville, Ontario, Canada) supplied the radioactive reagents and NEN Canada (Lachine, P.Q., Canada) the Aquasol-2 for scintillation counting. Unlabelled Dnp-F, phenylalanine and L-β-imidazole-lactic acid were obtained from Sigma, thin-layer silica gel plates were from Eastman-Kodak and Spectra-/Por 3 dialysis tubing from Spectrum Medical Industries (Los Angeles, CA, U.S.A.). Canlab (Ottawa, Ontario, Canada) supplied the following tubes: Pyrex (Corning; 16mm × 125mm); polystyrene (Kimble; 16mm × 125mm); and Tefzel ETFE (Nalgene; 13mm × 100mm).

Sample preparation

For labelling with acetic anhydride, a stock solution containing equimolar (3mM) insulin with phenylalanine was prepared in 5mM-N-methylmorpholine/5mM-sodium borate/0.1M-KCl adjusted to pH4.1. For Dnp-F labelling, stock solutions of equimolar (3mM) insulin with L-β-imidazole-lactic acid were prepared in 5mM-sodium monohydrogen phosphate with the desired KCl concentration (0.1, 0.5 or 1M).

^3H trace-labelling

[^3H]Acetic anhydride. Aliquots of the stock solution containing insulin and phenylalanine were diluted to concentrations of 1×10^{-5} and 5×10^{-6}M, in Pyrex tubes adjusted to desired pH value with 1M-KOH and temperature-equilibrated at 37°C in a thermostated water jacket. An aliquot of [^{3}H]acetic anhydride in 50μl of acetonitrile (1.24nmol, sp. radioactivity 6.7Ci/mmol) was added to a 5.0ml sample of the above with rapid stirring. The reaction was allowed to proceed for 5min and then concentrated HCl was added to bring the pH to 2 [Fig. 1, (2)]. After addition of 5g of urea, the pH was adjusted to 9 and the insulin fully acetylated with unlabelled acetic anhydride (50μl) at 22°C [Fig. 1, (3)].

[^3H]Dnp-F. Aliquots of the stock solution containing insulin and L-β-imidazole-lactic acid were diluted to concentrations of 1×10^{-3}, 5×10^{-4}, 1×10^{-4}, 5×10^{-5}, 1×10^{-5}, 5×10^{-6}, 1×10^{-6}, 5×10^{-7} and 1×10^{-7}M in appropriate test tubes, adjusted to pH7.5 with 1M-KOH and maintained at 37°C in a water bath. To each 2.5ml aliquot of the above 25μl of acetonitrile containing [^{3}H]Dnp-F (2nmol, sp. radioactivity 12.5Ci/mmol) was added and the reaction was allowed to proceed for 18h in the dark. Concentrated HCl was added to bring the pH to 2 [Fig. 1, (2)]. After addition of 2.5g of urea and 0.25g of $NaHCO_3$, each sample was fully dinitrophenylated with unlabelled Dnp-F [50μl, 50% (v/v) in acetonitrile] for a further 18h at 22°C in the dark [Fig. 1, (3)].

^{14}C labelling

[^{14}C]Acetic anhydride. A portion of the stock solution containing 15μmol of insulin and phenylalanine was lyophilized and then dissolved in 10ml of 0.1M-sodium borate/8M-urea. The sample was fully acetylated at pH9, using a pH-stat, with 750μmol of [^{14}C]acetic anhydride (sp. radioactivity 333mCi/mol). The final volume was adjusted to 15ml, and 0.5ml was added to each ^{3}H-labelled sample [Fig. 1, (5)], and the pH was lowered to 2.

[^{14}C]Dnp-F. A portion of the stock solution containing 2μmol of insulin and L-β-imidazole-lactic acid was lyophilized and then dissolved in 2ml of a solution of 8M-urea containing 2.5g of $NaHCO_3$. The sample was fully dinitrophenylated with 31.3μmol of [^{14}C]Dnp-F (sp. radioactivity 200mCi/mol) by reacting for 18h at 22°C. The final volume was adjusted to 25ml and 2.0ml was added to each ^{3}H-labelled sample [Fig. 1, (5)]. After addition of 5mg of Dnp-insulin and 0.5mg of imidazole-Dnp-lactic acid to each sample (as carrier), the pH was adjusted to 2.

Isolation and purification of $^3H/^{14}C$-labelled derivatives

[^3H,^{14}C]Acetic anhydride. Samples from step 5 (Fig. 1) were extracted three times with 5ml of ethyl acetate. The ethyl acetate phase, which contained the [^{3}H,^{14}C]acetylated phenylalanine, was purified as previously described (Sheffer & Kaplan, 1979).

The aqueous phase, which contained the derivatized insulin (usually precipitated at the interface) was dialysed against several changes of distilled water using dialysis tubing with an M_r cut-off of 3500. After lyophilization, the insulin was dissolved in 250μl of formic acid and a performic acid oxidation was performed (Hirs, 1956). Peptides containing each uniquely labelled group were isolated from elastase digests as previously described (Sheffer & Kaplan, 1979).

[^{3}H,^{14}C]Dnp-F. The solution from step 5 (Fig. 1) was extracted with several volumes of diethyl ether to remove excess dinitrophenol and aid in precipitation of protein. Centrifugation at 1000*g* for 10 min separated the supernatant, which contained the [^{3}H,^{14}C]Dnp-imidazole-lactic acid from the [^{3}H,^{14}C]protein. The precipitate from the aqueous phase was washed twice with 0.5 ml of water. After adding these washings to the supernatant phase, the internal standard was purified by using the method of Chan *et al.* (1981).

pH Measurements and titrations

A Radiometer pH meter 26 fitted with a type GK 2321C glass electrode was used for pH measurements. Titrations were performed by adding titrant from an Agla micrometer syringe apparatus.

Liquid scintillation counting

All samples were dissolved in 50 μl of 0.01 M-HCl and added to 10 ml of Aquasol-2. Scintillation counting was performed on a programmable LKB 1215 RackBeta scintillation counter equipped with automatic quench correction and a disintegrations per minute converter.

Results

Table 1 gives the reactivity obtained for the amino groups (glycine-A1, phenylalanine-B1 and lysine-B29) using acetic anhydride with phenylalanine as internal standard at 37°C in Pyrex glass tubes for a series of pH values. The notable feature in these data is the decrease of approximately an order of magnitude in reactivity of the two *N*-termini at the lower concentrations, especially in the physiological pH range. A more extensive study of the concentration-dependence of reactivity using Dnp-F with imidazole-lactic acid as internal standard (Fig. 2) at pH 7.5 shows that this large decrease in reactivity in the amino groups occurs at a concentration between 5×10^{-5} and 1×10^{-5} M. It is observed that while the reactivity of the amino groups remains constant down to a concentration of 5×10^{-5} M, the average reactivity of the imidazole groups (B5 and B10) decreases over the entire range of concentrations. At the lowest concentrations studied (1×10^{-6}–1×10^{-7} M), all the functional groups are non-reactive.

In order to investigate the possibility that adsorption of insulin to the containing vessel occurs at low concentration, the concentration dependence was studied under identical reaction

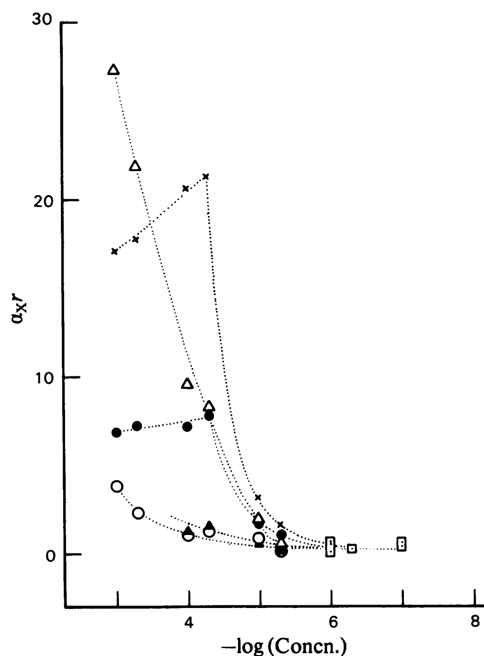

Fig. 2. *Concentration–reactivity profiles of the functional groups of insulin in Pyrex glass vessels*
Reagent, Dnp-F; solvent, 0.1 M-KCl, 37°C, 5 mM-phosphate, pH 7.5. ●, glycine; ×, phenylalanine; ○, lysine; ▲, tyrosine; △, histidine. ⊡ indicates data points of too close proximity to be differentiated graphically.

Table 1. *Competitive labelling of pig zinc insulin with acetic anhydride at 37°C in Pyrex glass*

Amino group	Concentration (μM)	$10^3 \times \alpha_x r$ pH ... 6.55	6.75	7.00	7.25	7.50	7.75	8.00
Gly-A1	200*	2.96	11.5	6.79	215	293	307	315
	10	0.397	2.97	6.72	12.0	21.0	36.7	58.3
	5	0.512	2.44	5.54	7.03	18.9	31.1	43.6
Phe-B1	200*	9.31	23.3	155	335	311	175	162
	10	1.61	4.40	6.93	8.98	10.7	13.5	14.7
	5	0.493	3.29	4.55	5.32	9.76	9.65	13.6
Lys-B29	200*	0.422	0.926	7.00	21.6	25.4	22.4	31.6
	10	1.13	1.83	2.11	3.64	6.24	10.7	15.7
	5	0.366	0.957	1.19	2.52	6.20	6.76	16.1

* Sheffer & Kaplan (1979).

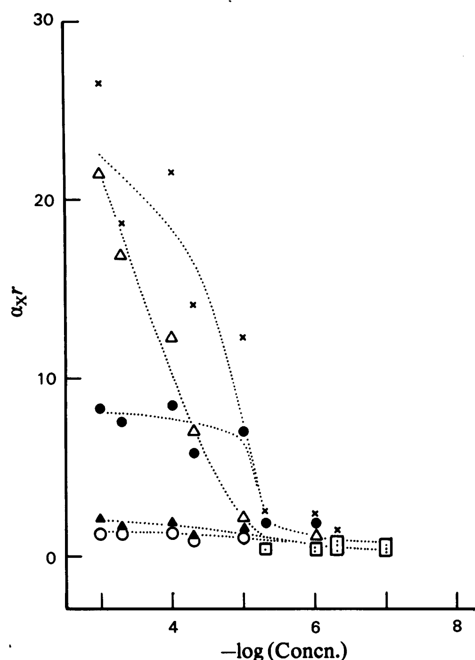

Fig. 3. *Concentration–reactivity profiles of the functional groups of insulin in Tefzel vessels*
Reagent, Dnp-F; solvent, 0.1 M-KCl, 37°C, 5 mM-phosphate, pH 7.5. ●, glycine; ×, phenylalanine; ○, lysine; ▲, tyrosine; △, histidine. ⊡ indicates data points of too close proximity to be differentiated graphically.

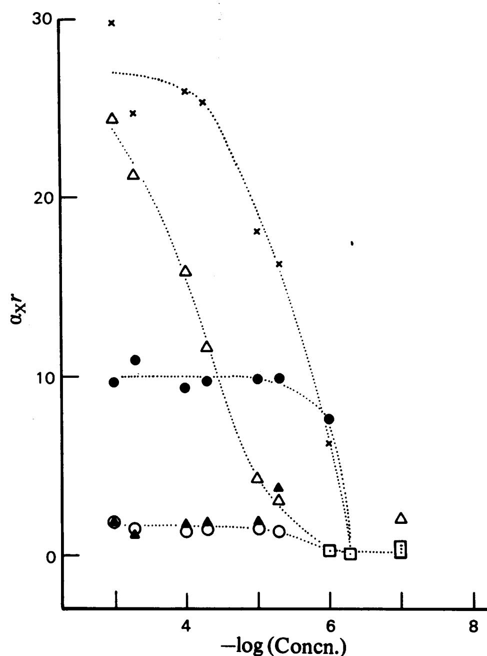

Fig. 4. *Concentration–reactivity profiles of the functional groups of insulin in polystyrene vessels*
Reagent, Dnp-F; solvent, 0.1 M-KCl, 37°C, 5 mM-phosphate, pH 7.5. ●, glycine; ×, phenylalanine; ○, lysine; ▲, tyrosine; △, histidine. ⊡ indicates data points of too close proximity to be differentiated graphically.

conditions as above, but in Tefzel (an analogue of Teflon fluorocarbons) tubes (Fig. 3) and polystyrene tubes (Fig. 4). The results obtained demonstrate that the glycine *N*-terminus remains reactive down to a much lower concentration than in Pyrex glass (Tefzel to 1×10^{-5} M and polystyrene to 1×10^{-6} M). However, the phenylalanine *N*-terminus exhibits a more gradual decrease over these concentration ranges, while the histidine residues show a very similar concentration dependence in the three types of tubes. Again at very low concentrations (1×10^{-7} M) all the groups are non-reactive.

Fig. 6 shows the effect of 1 M-salt on the concentration dependence of reactivity in Pyrex glass containers. The reactivity of the glycine *N*-terminus remains constant at all concentrations down to 1×10^{-7} M. The phenylalanine *N*-terminus shows a substantial decrease below 5×10^{-6} M. Again the histidine results show a decline in reactivity at all concentrations but, while the absolute reactivities of the *N*-termini remain approximately the same as in 0.1 M-salt, the average histidine reactivity has more than doubled. Also, in contrast with 0.1 M-salt, all the groups are reactive at a concentration of 1×10^{-7} M. The results obtained in 0.5 M-salt

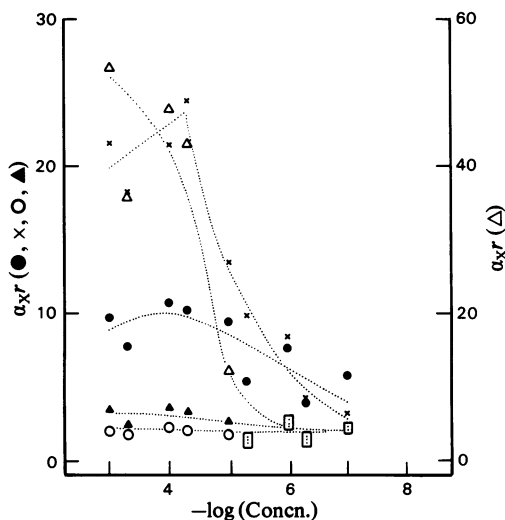

Fig. 5. *Concentration–reactivity profiles of the functional groups of insulin in Pyrex glass vessels*
Reagent, Dnp-F; solvent, 0.5 M-KCl, 37°C, 5 mM-phosphate, pH 7.5. ●, glycine; ×, phenylalanine; ○, lysine; ▲, tyrosine; △, histidine. ⊡ indicates data points of too close proximity to be differentiated graphically.

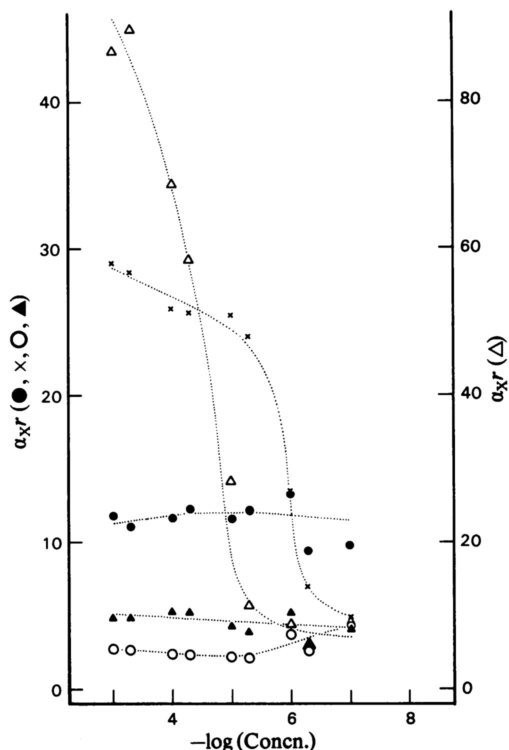

Fig. 6. *Concentration–reactivity profiles of the functional groups of insulin in Pyrex glass vessels*
Reagent, Dnp-F; solvent, 1.0M-KCl, 37°C, 5mM-phosphate, pH7.5. ●, glycine; ×, phenylalanine; ○, lysine; ▲, tyrosine; △, histidine. ⊡ indicates data points of too close proximity to be differentiated graphically.

(Fig. 5) are intermediate to those obtained in 0.1M-salt and 1M-salt.

Discussion

Chemical modification has been widely applied as an approach to the study of the structure and function of proteins (Cohen, 1970; Glazer, 1976). Previous studies with competitive labelling have demonstrated that this approach can be used not only to determine the chemical properties of groups but also as a sensitive probe for conformational change (Kaplan *et al.*, 1982; Kraal & Hartley, 1978; Bresciani, 1977). In the case of proteins such as insulin, the free monomeric form can be studied only at very low concentrations. However, at present, there are considerable technical difficulties in the study of proteins at very low concentrations by physical methods (Blundell & Wood, 1982). Our results show that the described modifications to the competitive labelling procedure make it possible to determine the chemical properties and, from these, to infer structural properties of proteins at high dilutions. In this initial application the lowest concentration employed was 1×10^{-7}M; however, we are currently able to carry out studies at concentrations as low as 1×10^{-9}M.

Insulin exhibits a complex, concentration-dependent association pattern (Blundell *et al.*, 1972). Various models have been proposed to account for the observed apparent weight-average molecular weight obtained in sedimentation equilibrium studies (Milthorpe *et al.*, 1977; Goldman & Carpenter, 1974; Pekar & Frank, 1972; Jeffrey & Coates, 1966). All models allow for free monomer, dimer and hexamer with the possibility of higher molecular weight forms. In these studies the lowest concentration employed was approx. 1×10^{-5}M, and a dimer dissociation constant of the order of 10^{-5}M was determined. On the basis of these studies, it would appear that at neutral pH values the free monomer form of insulin predominates at concentrations below 1×10^{-6}M. At higher concentrations determination of the proportions of associated forms, i.e. dimer, tetramer, hexamer and higher forms, is dependent on the particular association model chosen for analysis of the sedimentation data.

In the concentration range where associated forms of insulin predominate, several of the functional groups have unusual chemical properties (Chan *et al.*, 1981; Sheffer & Kaplan, 1979). The *N*-termini, the ε-amino group of lysine-B29 and the histidine residues are abnormally reactive at physiological temperature and pH. In the case of the phenylalanine *N*-terminus, its high reactivity is coupled with an unusually low p*K* value. The present study not only confirms the high reactivities observed previously but also shows that the reactivity of some functional groups in insulin is concentration-dependent. The fact that at high dilution an extremely low reactivity was observed toward two reagents and different internal standards, i.e. acetic anhydride with phenylalanine and Dnp-F with L-β-imidazole-lactic acid, which have substantially different chemical and physical properties, demonstrates that the observed decreases in reactivity are unlikely to be due to specific interactions of the labelling reagents with insulin in any of its forms.

Previous studies on insulin (Chan *et al.*, 1981; Sheffer & Kaplan, 1979) and the initial experiments in this study (Table 1) were carried out in Pyrex glass reaction vessels. The data reported in Table 1 represent an attempt to obtain the pH dependence of the reactivity of the amino groups in the free insulin monomer by using acetic anhydride as the chemical probe and to derive the chemical properties of the individual functional groups. An examination of the data shows that the amino

groups do not exhibit the titration behaviour of free amines in solution. Even more notable was the observation that, while the reactivity of the groups was very high at a concentration of 2×10^{-4} M, the reactivity at 1×10^{-5} M and at 5×10^{-6} M was extremely low. The question that arises is whether this observed decrease reflects a change in reactivity on formation of the free monomer or adsorption of protein to the reaction vessel. Adsorption of insulin to glass vessels has previously been reported (Hollenberg & Cuatrecasas, 1976) but the extent of adsorption was not quantified. In order to answer the question as to whether adsorption was having a significant effect on the reactivity data, reactions were carried out in vessels made of Pyrex glass (Fig. 2), Tefzel (Fig. 3) and polystyrene (Fig. 4). Dnp-F was used as the chemical probe because it is possible to obtain data for tyrosine and histidine groups in addition to amino groups and also provide evidence for any specific effects due to the labelling reagents.

The data obtained with Pyrex glass vessels (Fig. 2) parallels the results obtained with acetic anhydride (Table 1). At concentrations where associated forms predominate, the reactivities of the amino groups are much higher than at the lower concentrations where the free monomer is expected to predominate. Below 1×10^{-6} M, all groups become non-reactive, which is very suggestive of adsorption to the surface. In addition, the reactivities of both the *N*-termini simultaneously exhibit a sharp drop in reactivity at approx. 5×10^{-5} M, which is consistent with an adsorption phenomenon. On the other hand, the reactivity of the histidine residues is decreasing markedly even at concentrations greater than 5×10^{-5} M, while the reactivities of the *N*-termini are unchanged. This indicates that there is an alteration in the microenvironment of the histidine residues in the associated monomeric unit which is dependent on the degree of association. The lysine and tyrosine groups have relatively low reactivities at all concentrations so that the decrease in reactivity on adsorption is not as marked as in the other groups.

The data obtained in polystyrene vessels (Fig. 4) show a distinctly different pattern of relative reactivities for the *N*-termini but not the other functional groups. Most notably, the reactivity of the glycine *N*-terminus is unaffected at concentrations as low as 1×10^{-6} M, which is in sharp contrast to Pyrex glass, where the reactivity all but vanishes at 1×10^{-5} M (Fig. 2). The data for the phenylalanine *N*-terminus, however, parallels that with Pyrex glass in that the reactivity is constant at concentrations above 5×10^{-5} M and declines at lower concentrations, but the decrease is much more gradual so that at 1×10^{-6} M there is still an appreciable reactivity. Below 1×10^{-6} M there is a sharp decrease in reactivity in all groups and, as in the case of Pyrex glass, all the reactivities approach zero. The decrease in reactivity of the histidine residues on dilution appears to be identical in both types of reaction vessel. In the case of Tefzel vessels (Fig. 3), the change in reactive properties of the *N*-termini on dilution is intermediate to that in glass and polystyrene, and again the histidine residues show the same decline at concentrations where the other groups have constant reactivities.

It appears that at very low concentrations the bulk of the insulin is adsorbed to the surface of the reaction vessels. This conclusion is based on the observations that on dilution all functional groups become non-reactive in all three types of reaction vessels but at different concentrations (5×10^{-5} M with glass, 1×10^{-5} M with Tefzel and 1×10^{-6} M with polystyrene). The concentration-dependence of the reactivity of the histidine residues in all types of reaction vessel (Figs. 2, 3 and 4) and the phenylalanine *N*-terminus in polystyrene (Fig. 4) cannot be explained solely on the basis of an adsorption effect in which the reactivities of all groups should decrease simultaneously. These results therefore indicate that the microenvironments of the histidine residues and phenylalanine *N*-terminus are dependent on the degree of association.

In the case of Pyrex glass where the adsorption is the strongest, this property is probably due to charge interaction between the glass surface and the insulin. Increasing the salt concentration should minimize this interaction and perhaps provide conditions where no adsorption takes place. At higher salt concentrations (0.5 M in Fig. 5, and 1 M in Fig. 6), the results obtained at insulin concentration greater than 1×10^{-6} M clearly parallel those obtained with 0.1 M-salt in polystyrene reaction vessels. Although some adsorption effects are evident in 0.5 M-salt, all the functional groups retain significant reactivity at an insulin concentration of 1×10^{-7} M. This indicates that the adsorption effects are not nearly as strong as in 0.1 M-salt (Fig. 2). In the case of 1 M-salt it appears that adsorption effects are no longer significant, since all functional groups retain at least normal reactivity at all concentrations. The reactivity of glycine remains constant at all concentrations and the abnormally high reactivity of the histidine residues has decreased to a normal value at a concentration of 1×10^{-5} M. The reactivity of the phenylalanine *N*-terminus decreases to a value below that of the glycine but is still in a range that can be considered normal for such a group. In contrast with the other groups, the reactivity of the solitary lysine residue increases at concentrations where the free monomer predominates. High salt concentration has been reported to substantially increase the affinity of insulin for its receptor (Tatnell & Jones, 1981)

indicating that the properties observed are for a functional insulin monomer.

The reactivity data obtained in this study clearly show that the microenvironment of some functional groups changes as the state of association of insulin goes from hexamer to dimer to monomer. The question is whether these changes reflect a difference in the conformation of the monomeric unit in the free and associated states or differences due to disruption of intermonomer interactions in the associated states. If a major conformational change occurs when the associated monomeric unit dissociates to the free form, then one would expect simultaneous changes in the reactivities of all functional groups. The histidine residues only decrease in reactivity as the concentration decreases from 1×10^{-3} to 5×10^{-6} M. Based on the sedimentation studies, this is in the concentration range where hexamers and possibly tetramers are dissociating to form dimers. In the concentration range 5×10^{-6}–1×10^{-7} M where the dimer is dissociating to form the free monomer, the reactivity is constant. The phenylalanine *N*-terminus and lysine have the opposite reactivity pattern. The reactivity remains unchanged in the transition from hexamer to dimer, but on free monomer formation the reactivity increases in the case of lysine and decreases in the case of phenylalanine. These observations therefore indicate that the changes in reactivity of the histidine residues reflect alterations in their microenvironment on dimer–dimer association and for phenylalanine and lysine on monomer–monomer association. The reactivity of the glycine *N*-terminus and the average reactivity of the tyrosine residues are independent of concentration, indicating that the environments of these groups are essentially the same in all states of association.

The only approach, other than that reported here, that has been used to investigate the conformation of the insulin monomer is c.d. Wood *et al.* (1975) have interpreted their results as showing little or no change in conformation between the free monomeric unit and the monomeric unit in the dimer. More recently Pocker & Biswas (1980), using improved instrumentation, claim that the transition from dimer to monomer is accompanied by substantial changes in helix, β-sheet and random coil content in the monomeric unit. The reactivity data obtained in this study show that the changes that occur in microenvironment are due to interactions between the monomeric units in the associated forms and that there is little or no conformational difference in the monomeric unit in its associated or free form. Blundell & Wood (1982) have implied that surface adsorption may have affected the data reported by Pocker & Biswas (1980). On the basis of our results which show strong adsorption to various materials, this appears to be a possible explanation for the apparent change in the spectra they obtained at low concentrations.

On the basis of X-ray crystallographic studies several functional groups of insulin have been postulated to be involved in either inter- or intramonomer interactions (Chothia *et al.*, 1983; Blundell *et al.*, 1971). Of particular interest to this study are those involving the histidine residues. The zinc content of the insulin used in the present study corresponds to that of the 2 Zn–insulin crystals. In this structure, the B10 residue of each monomeric unit is involved in binding the zinc. Therefore, the abnormally high reactivity of the histidines in associated states of insulin observed here and in a previous study (Chan *et al.*, 1981) cannot be due to this particular residue. The B5 residue is postulated to be involved in an intramonomer hydrogen bond through the N^{δ} to the peptidyl carbonyl of A7 and an intermonomer hydrogen bond through the N^{τ} to the backbone of the A1 helix of an adjacent hexamer. The N^{δ} interaction should lead to enhanced reactivity as postulated previously (Cruickshank & Kaplan, 1972; James, 1980) to explain the enhanced reactivity observed for the active centre histidine of chymotrypsin. The N^{τ} interaction, however, should lead to decreased reactivity, since this is the only position at which Dnp-F can react (Henkart, 1971). Neither of these postulated interactions in the crystal explains our observations. Our data indicate that, in solution, the N^{δ} is involved in hydrogen bonding only in dimer–dimer interactions, whereas in the dimer and free monomer the histidines have a normal reactivity and therefore are apparently not involved in any strong hydrogen bonding. In 1 M-salt the reactivity of the histidine in the hexameric or tetrameric form of insulin is further increased. This cannot be due to a specific imidazole effect, since imidazolelactate is the internal standard, but may reflect a strengthening of hydrogen bonding in 1 M-salt.

The α-amino group of the A1 glycine occupies a surface pocket in the crystal. Its accessibility does not appear to depend on the degree of association, which is in complete accord with our findings. The corresponding amino group of the B1 phenylalanine is postulated to be in position to interact with the A17 carboxyl of an adjacent dimer in the crystal. Our data does not indicate such a dimer–dimer interaction in solution but shows that this group is involved in a monomer–monomer interaction leading to enhanced reactivity. Unfortunately, the position of the B29 lysine ε-amino group is not well defined in the crystal structure. Our reactivity data indicate that, like the phenylalanine *N*-terminus, it is involved in a monomer–monomer interaction.

Current evidence indicates that the insulin monomer associates with its membrane receptor and with itself primarily through hydrophobic interactions with several residues common to both associations (Blundell & Wood, 1982; Pullen *et al.*, 1976). Our data show that association has the effect of dramatically increasing the apparent nucleophilicity of the histidine residues and the phenylalanine *N*-terminus. It has been our experience that functional groups with unusual chemical properties in proteins have a crucial role; for example, the active centre histidine of chymotrypsin (Cruickshank & Kaplan, 1972), the *N*-termini of the pancreatic serine proteinases (Kaplan, 1972; Kaplan *et al.*, 1971) and the *N*-termini of haemoglobin (Kaplan *et al.*, 1982; Garner *et al.*, 1975). It may be coincidental but the properties of the phenylalanine *N*-terminus of insulin in its associated states closely resemble those of the valine *N*-termini of haemoglobin in terms of unusually low p*K* and abnormally high reactivity. Indeed it has been shown that this group, like those of haemoglobin, readily reacts with CO_2 (Sheffer & Kaplan, 1979; Kilmartin & Rossi-Bernardi, 1973). It is intriguing to speculate that on binding of the free insulin monomer to the receptor the unusual chemical properties which are present only in the associated forms are restored and that they are involved in initiating the hormone's action or modulating its activity.

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