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Structure–function relationships in the free insulin monomer

Mary A. HEFFORD, George ODA and Harvey KAPLAN*
Department of Biochemistry, University of Ottawa, Ottawa, Ont. K1N 9B4, Canada

The chemical properties of the functional groups of insulin were determined at a concentration (0.5 μM) where the predominant species of insulin is the free (unassociated) monomeric unit. The glycine *N*-terminus and the four tyrosine phenolic groups had the same properties as in the associated forms of insulin. On the other hand the lysine ϵ-amino group and the two histidine imidazole groups had substantially altered properties. Some alteration in the properties of the phenylalanine *N*-terminus was also observed. The reactivity–pH profile for the imidazole groups showed a second ionization with a pK_a of 10.1 in addition to an ionization with a pK_a of 6.8. On the basis of the X-ray-crystallographic structure of hexameric insulin the observed changes can be accounted for by disruption of monomer–monomer or dimer–dimer interactions in the associated states of insulin. It is concluded that the conformation of the monomeric unit of insulin is essentially the same in its free and associated states in solution.

INTRODUCTION

The free monomeric unit of insulin is currently believed to be a biological species relevant to receptor binding (Blundell, 1979; Chothia *et al.*, 1983; Hodgkin *et al.*, 1983). An important question is whether the conformation of this species in solution is similar to or different from that of the monomeric unit in the hexameric form as determined by X-ray crystallography. There is very little direct evidence in the literature on this point, but the data that do exist (Wood *et al.*, 1975; Chothia *et al.*, 1983; Kaplan *et al.*, 1984) indicate that the structures are probably very similar. The evidence, however, is far from conclusive on this point, since evidence for substantial differences has been reported (Pocker & Biswas, 1980). The present study was undertaken with the objective of determining the chemical properties, namely pK_a and reactivity, of the functional groups of the free insulin monomer in an attempt to answer this question.

The method of competitive labelling (Duggleby & Kaplan, 1975; Kaplan *et al.*, 1971) provides an experimental approach for determining the chemical properties (namely pK_a and reactivity) of functional groups in proteins. A feature of this method is that the reaction with radiolabelled reagent is carried out under conditions such that the fraction of any group that reacts is negligible in comparison with the total present. The labelled reagent therefore always reacts with the native (i.e. unmodified) protein and the chemical properties obtained must apply to the native protein. Another crucial feature is that the labelled groups of interest are isolated and identified, so that the assignment of parameters to specific groups is unequivocal. The inclusion of an internal standard of known pK_a and reactivity that competes with the reagent permits the determination of these parameters for the groups of interest in the native protein.

The study of the free insulin monomer is made difficult by the fact that the dimer dissociation constant is approx. 10^{-5} M (Jeffrey & Coates, 1966; Pekar & Frank, 1972; Goldman & Carpenter, 1974; Milthorpe *et al.*, 1977). This means that studies on the free monomer must be carried out at concentrations below 1 μM. An added complication is the fact that insulin in dilute solution strongly adsorbs on glass surfaces (Hollenberg & Cuatrecasas, 1976; Kaplan *et al.*, 1984). Recent improvements in the competitive labelling methodology (Kaplan *et al.*, 1984; Hefford *et al.*, 1985) have made it possible to determine the reactivity of functional groups of proteins in extremely dilute solutions, so that the study can be carried out at a concentration (0.5 μM) where the free insulin monomer predominates. The problem of adsorption can be eliminated by carrying out the study in 1 M-salt, where insulin does not adsorb on glass surfaces (Kaplan *et al.*, 1984).

MATERIALS AND METHODS

Materials

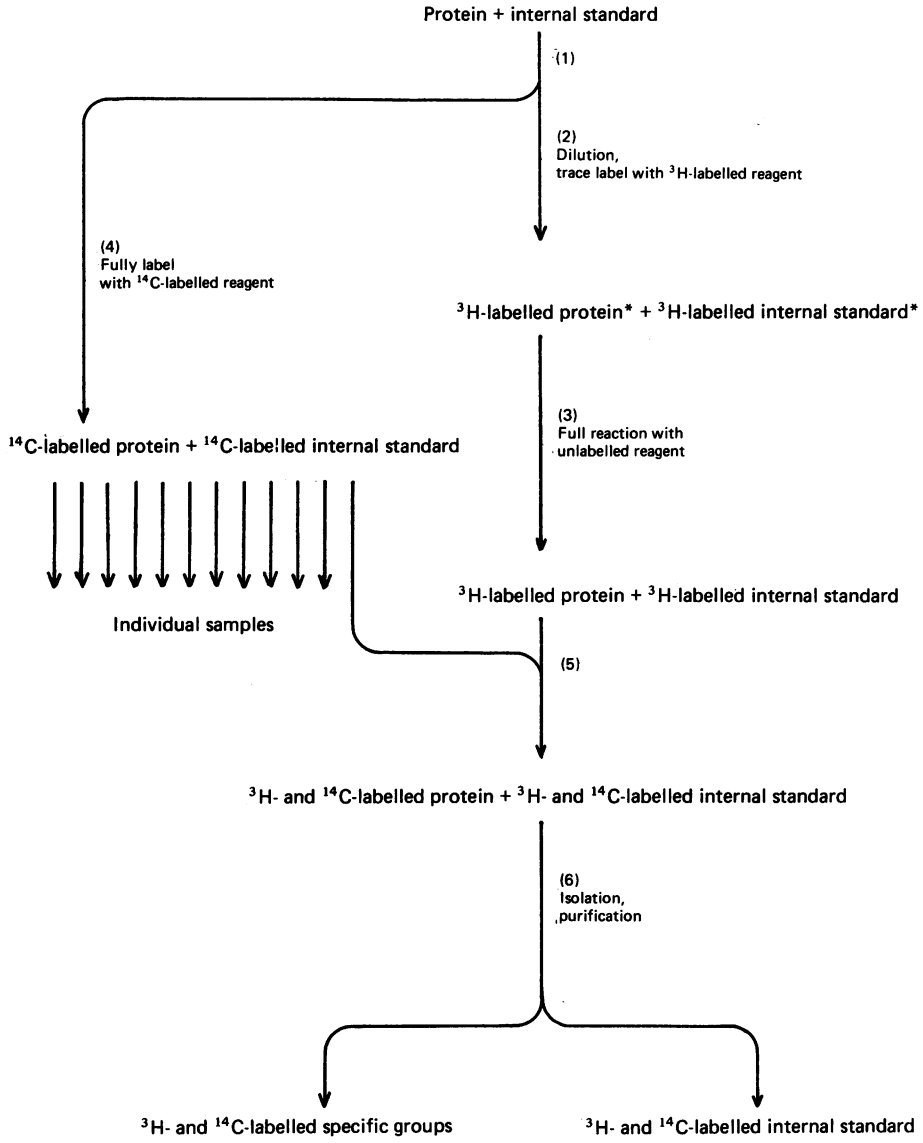
Pig zinc insulin (0.35% zinc by wt.) was donated by Connaught Laboratories (Toronto, Ont., Canada). Fine chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), radioactive reagents were from Amersham Corp. (Oakville, Ont., Canada) and Aquasol-2 was from NEN Canada (Lachine, P.Q., Canada). The Eastman–Kodak thin-layer silica-gel plates and Spectra/Por 3 dialysis tubing were supplied by Fisher Scientific (Ottawa, Ont., Canada).

Sample preparation

A stock solution containing equimolar amounts (5 μmol) of insulin and alanylalanine was prepared and adjusted to pH 4.0. A portion was added to a buffer consisting of 5 mM-*N*-methylmorpholine/5 mM-acetic acid/5 mM-sodium borate/1.0 M-KCl to give a final insulin concentration of 0.5 μM [Scheme 1, step (1)]. This solution was used for the trace-labelling with ^{3}H, with the remainder of the stock solution being used in labelling with ^{14}C.

Abbreviation used: Dnp, 2,4-dinitrophenyl.

* To whom all correspondence should be addressed.



Scheme 1. Flow diagram of experimental procedure

For full experimental details see the text.

Trace-labelling with ^{3}H

Portions (2.5 ml) of the 0.5 μM-insulin solution were equilibrated at 37 °C and adjusted to the desired pH with either 1 M-KOH or 1 M-HCl. To each sample was added 50 μl of acetonitrile containing [^{3}H]Dnp-F (1.20 nmol; sp. radioactivity 16.6 Ci/mmol) and the reaction allowed to proceed for 18 h at 37 °C in the dark [Scheme 1, step (2)]. The pH was then adjusted to 2 with conc. HCl. After the addition of 2.5 g of urea and 0.5 g of $NaHCO_3$ to each sample, complete dinitrophenylation was assured by reaction for a further 18 h in the dark at 37 °C with 100 μl of unlabelled Dnp-F [50% (v/v) in acetonitrile] [Scheme 1, step (3)].

Labelling with ^{14}C

The remaining portion of the stock solution (99 ml) was freeze-dried and redissolved in 6 ml of 8 M-urea (cyanate-free). After the addition of 1.0 g of $NaHCO_3$, dinitro[^{14}C]phenylation was accomplished by reaction with 0.5 mmol of [^{14}C]Dnp-F (sp. radioactivity 250 Ci/mmol) for 18 h at 22 °C in 8 M-urea solution, and 0.5 ml of this resulting solution was added to each ^{3}H-trace-labelled sample [Scheme 1, step (5)].

Isolation and purification of [^{3}H,^{14}C]Dnp derivatives

The solutions from step (5) (Scheme 1) were extracted with diethyl ether (4 × 5 ml) to remove [^{3}H,^{14}C]Dnp-alanylalanine and dinitrophenol as well as to aid in the precipitation of the protein. The [^{3}H,^{14}C]Dnp derivatives were isolated from the precipitated protein solution after hydrolysis in 6 M-HCl and purified as described previously (Chan *et al.*, 1981). The [^{3}H,^{14}C]Dnp-alanylalanine was separated from dinitrophenyl by the method of Steven (1962) and purified by t.l.c. (Kaplan *et al.*, 1978) with a further silica-gel G t.l.c. with chloroform/benzyl alcohol/acetic acid (70:30:3, by vol.) [Scheme 1, step (6)].

Liquid-scintillation counting

All samples were dissolved in 100–500 μl of 10 mM-HCl and added to 10 ml of Aquasol-2. Scintillation counting was performed on a programmable LKB 1215 Rack Beta scintillation counter equipped with automatic quench correction and a disintegrations-per-minute converter.

Calculation of reactivities

The reactivity ($\alpha_x r$) at each pH value was calculated from the following equations (Kaplan *et al.*, 1971; 1984):

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

$$\alpha = 1\Big/\left(1+\frac{[H^+]}{K_a}\right)$$

where α_x is the degree of ionization of the functional group being studied, α_s is the degree of ionization of the internal standard, and r is the pH-independent second-order velocity constant for reaction of the functional group relative to that of the internal standard. The experimental determined parameters are the $^3H/^{14}C$ radioactivity ratios, $(^3H_x/^{14}C_x)$ and $(^3H_s/^{14}C_s)$, for the group of interest and internal standard respectively. A plot of the $\alpha_x r$ values versus pH gives a sigmoidal titration curve. The pH value at the inflexion point is equal to the pK_a of the group under study and the limiting value at high pH is equal to r.

RESULTS

A technical point of interest with regard to the internal standard alanylalanine should be noted. Previous studies (Chan *et al.*, 1981; Kaplan *et al.*, 1984) used imidazol-3-yl-lactate as an internal standard. It was found that the Dnp-imidazole derivative slowly breaks down in light. Although this was not a problem at the high concentrations used in previous studies, it was considered that it would be a problem at the very low concentrations used in the present study. For this reason alanylalanine, which yields a very stable Dnp derivative, was used in the present study.

Fig. 1 shows a plot of $\alpha_x r$ against pH for the glycine *N*-terminus in the free monomer. The experimental points closely follow the theoretical titration curve with a pK_a value of 8.21 and reactivity of 2.22 relative to alanylalanine. Similar plots for the ϵ-amino group of the lysine at position B29 and the four tyrosine residues are shown in Figs. 2 and 3. Again the data lie very close to the theoretical titration curves with pK_a values of 9.80 ± 0.05 and 9.18 ± 0.05 and reactivities of 25.4 ± 1.9 and 4.45 ± 0.18 relative to alanylalanine.

The reactivity–pH profile for the two histidine residues is shown in Fig. 4. These groups exhibit normal titration behaviour below pH 8.25. When the pH is further increased, there is a substantial increase in the average reactivity, relative to the internal standard, of the two histidine residues towards Dnp-F, indicating that another ionization results in a very reactive species. Non-linear least-squares analysis of the data assuming two ionizations gives a pK_a value of 6.80 ± 0.26 and a reactivity relative to alanylalanine of 0.234 ± 0.033 for the first ionization and a pK_a value of 10.12 ± 0.50 and a reactivity of 2.1 ± 1.7 for the second. On excluding the data above pH 8.25, a very similar pK_a value, 6.78 ± 0.23, for the first ionization is obtained.

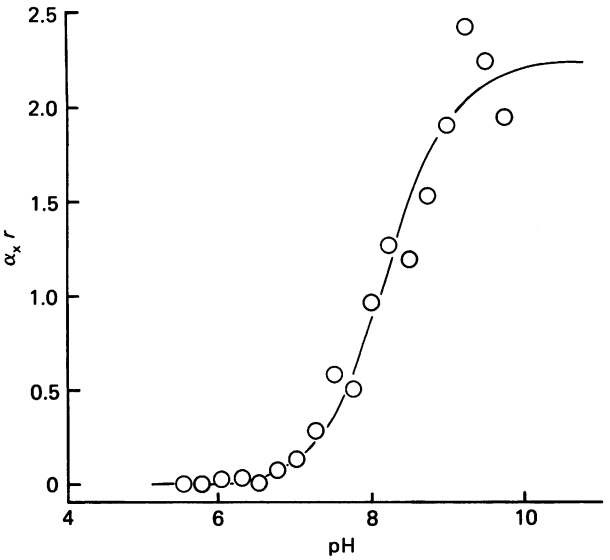


Fig. 1. Reactivity–pH profile of the glycine *N*-terminus of the free insulin monomer

The continuous line is a theoretical titration curve with a pK_a of 8.21 and an r of 2.22.

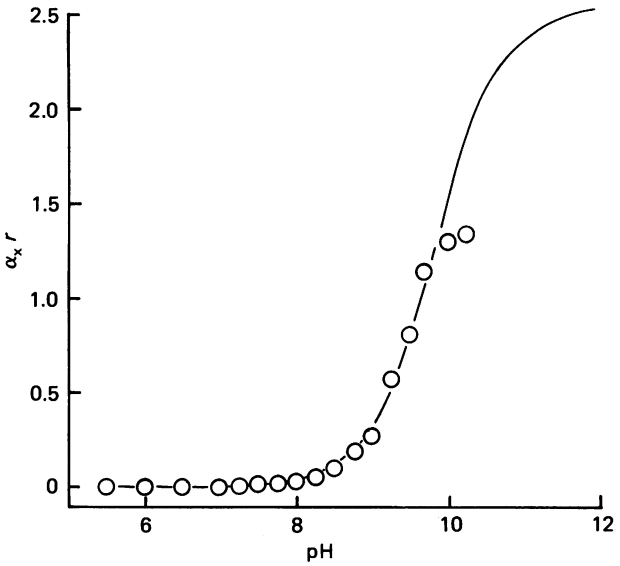


Fig. 2. Reactivity–pH profile of the ϵ-amino group of lysine-B29 of the free insulin monomer

The continuous line is a theoretical titration curve with a pK_a of 9.80 and an r of 25.4.

The change in reactivity of the phenylalanine *N*-terminus with pH was similarly analysed. Fig. 5 shows the fit of the experimental values to a theoretical titration curve with a pK_a of 6.92 and a reactivity of 0.644 relative to alanylalanine. It is clear that this functional group has an unusual reactivity–pH profile. Although low reactivity is observed below pH 7.0 and substantially higher reactivity above pH 7.0, the intermediate $\alpha_x r$ values expected at pH values near the pK_a value are absent.

If the internal standard, alanylalanine, is assumed to

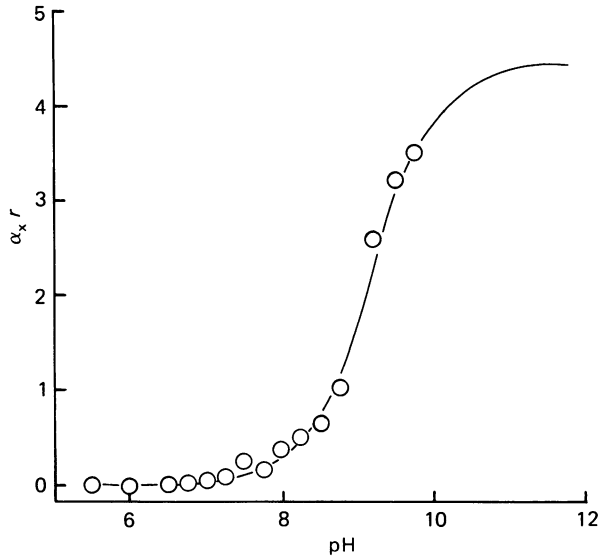


Fig. 3. Reactivity–pH profile of the four tyrosine residues of the free insulin monomer

The continuous line is a theoretical titration curve with a pK_a of 9.18 and an r of 4.45.

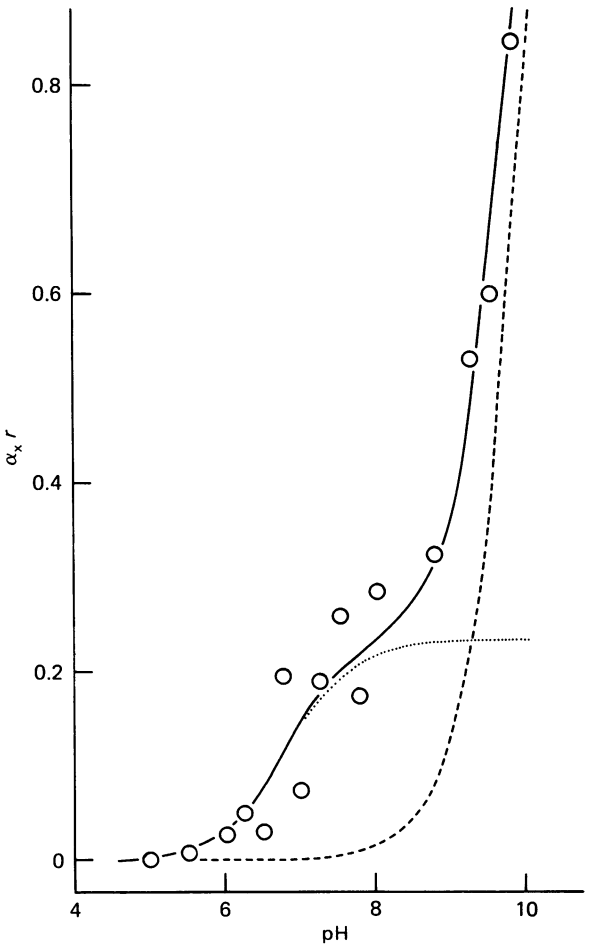


Fig. 4. Reactivity–pH profile of the two histidine residues of the free insulin monomer

The lines are theoretical titration curves with the following values: ·····, $pK_a = 6.80$, $r = 0.234$; -----, $pK_a = 10.1$, $r = 2.10$; ——, both curves together.

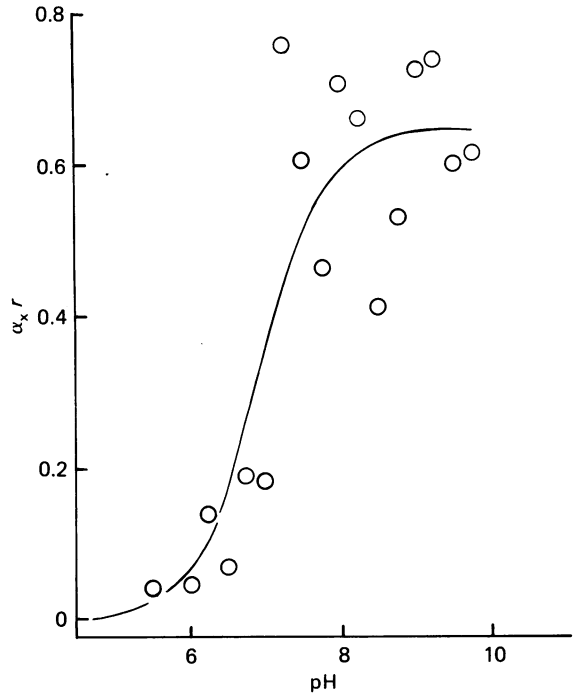


Fig. 5. Reactivity–pH profile of the phenylalanine *N*-terminus of the free insulin monomer

The continuous line is a theoretical titration curve with a pK_a of 6.92 and an r of 0.644.

lie on a Brönsted plot for the reaction of a series of standard amines with Dnp-F (Chan *et al.*, 1981), a second-order pH-dependent rate constant of 0.615 $M^{-1} \cdot min^{-1}$ is calculated. By using this value the second-order rate constants for the reactions of the functional groups in monomeric insulin with Dnp-F were estimated. Deviations from the Brönsted plot can be represented as relative reactivities (*RR*), i.e. the reactivity relative to that of a standard amine with the same pK_a value that lies on the plot (Kaplan *et al.*, 1971). Table 1 summarizes the pK_a values and relative reactivities obtained for the amino groups in insulin in its free monomeric (present study) and associated forms (Sheffer & Kaplan, 1979; Chan *et al.*, 1981). It is clear that these parameters are similar for the glycine *N*-terminus in all three studies but different for the lysine and phenylalanine amino groups. Second-order pH-dependent rate constants of 0.144 $M^{-1} \cdot min^{-1}$ and 2.7 $M^{-1} \cdot min^{-1}$ were calculated for the reaction of the two histidine and four tyrosine residues respectively and Dnp-F. Comparison of this parameter for the histidine imidazole function with those of standard imidazole groups (Cruickshank & Kaplan, 1975) shows that at least one of the two histidine residues in the insulin free monomer has higher than 'normal' reactivity. Comparison of the average rate constant for the four tyrosine residues ($k = 2.7$ $M^{-1} \cdot min^{-1}$) with that of the phenolic hydroxyl group of *N*-acetyltyrosine amide ($k = 29$ $M^{-1} \cdot min^{-1}$; Chan *et al.*, 1981) shows that these residues have a much lower-than-expected reactivity.

DISCUSSION

Previous competitive labelling studies of insulin at concentrations of 200 μM and 70 μM in 0.1 M-KCl (Sheffer

Table 1. Chemical properties of associated and free insulin monomers

RR is the relative reactivity (defined in the text).

			pK_a or *RR*		
		Concn. of insulin . . .	200 μM	70 μM	0.5 μM
		Labelling reagent . . .	Acetic anhydride	Dnp-F	Dnp-F
		Internal standard . . .	Phenylalanine	Imidazolyl-lactate	Alanylalanine
Functional group	Chemical property	Temperature . . .	10 °C	20 °C	37 °C
		Reference . . .	Sheffer & Kaplan (1979)	Chan *et al.* (1981)	Present study
Glycine	pK_a		7.9	7.7	8.21 ± 0.08
	RR		2.6	2.5	3.3
Phenylalanine	pK_a		7.2	6.9	6.92 ± 0.19
	RR		6.6	7.5	3.7
Lysine	pK_a		7.8	7.0	9.80 ± 0.05
	RR		0.023	1.7	3.9
Histidine	pK_a		—	6.5	6.80 ± 0.05
	RR		—	20	5.9
Tyrosine	pK_a		—	8.7	9.18 ± 0.05
	RR		—	0.08	0.09

& Kaplan, 1979; Chan *et al.*, 1981) demonstrate that, in the associated forms of insulin, the glycine *N*-terminus has a 'normal' pK_a and a 'normal' reactivity on the basis of a Brönsted plot. From the examination of concentration-dependence of reactivity at pH 7.5 (Kaplan *et al.*, 1984) one would predict that chemical properties of this group are the same in the free and associated states of the monomeric unit. Isolation of Dnp-glycine after competitive labelling of insulin with Dnp-F at 0.5 μM bears out this prediction since the relative reactivity determined is the same as in the associated forms of insulin (Table 1). Hence the microenvironment of the A-chain *N*-terminal glycine is very similar in the monomeric unit in its free and associated states.

The reactivity–pH profile of the four tyrosine residues of the free monomer (Fig. 3) shows a pK_a value of 9.18 ± 0.05 and a reactivity of 4.45 ± 0.18 relative to alanylalanine. Using the competitive-labelling procedure, Chan *et al.* (1981) estimated an average pK_a value of approx. 10 for the four tyrosine residues in insulin at a concentration of 68.6 μM, where the dimeric form predominates, a value that is slightly higher than that obtained in the present study. The average pK_a and reactivity values assigned to the tyrosine residues in associated forms of insulin by those authors, however, were merely estimated from a plot of the data. When these primary data were subjected to a regression analysis, an average pK_a value of 8.69 ± 0.04 was obtained for the four tyrosine residues in insulin in its associated forms. This latter value is only slightly lower than that of 9.18 ± 0.05 determined in the present study. These data indicate that there may be small differences in the average chemical properties of the tyrosine residues in the free monomeric and associated forms of insulin, but in both states the tyrosine residues are buried in similar environments.

The exact position of the ϵ-amino group of the lysine-B29 residue is not well defined in the crystal structure. It lies near the end of the extended *C*-terminal region of the B-chain on the outside of the hexamer. It is postulated that, in solution, the protonated ϵ-amino group is involved in a salt linkage with the carboxylate ion of the glutamate-44 residue of the same monomer (Blundell *et al.*, 1972). Such an interaction would result in the stabilization of the protonated ϵ-amino group relative to the deprotonated group and an increased pK_a for the lysine residue. In the free monomer the lysine-B29 ϵ-amino function has a pK_a value that is close to that expected for such a group in the absence of interactions. In a more concentrated insulin solution the lysine residue has an abnormally low pK_a value in the physiological pH range (Chan *et al.*, 1981). The postulated salt linkage involving the ϵ-amino group of the lysine-B29 residue in the free monomer is therefore not supported by its chemical properties.

The microenvironment of the *N*-terminal phenylalanine-B1 residue appears to change as monomers associate to form dimers, and no further change is seen as the dimer associates to form higher oligomers (Kaplan *et al.*, 1984). Previous studies (Bradbury & Brown, 1977; Sheffer & Kaplan, 1979; Chan *et al.*, 1981) were performed at concentrations above 50 μM, where, on the basis of sedimentation-equilibrium data, an equilibrium mixture of dimer and higher oligomers is expected. The change in the microenvironment of the B-chain *N*-terminus on monomer–monomer association is therefore expected to be apparent in a comparison of the chemical properties of this group in the free insulin monomer with those obtained in the previous studies of concentrated insulin solutions. In the free monomer the reactivity of this functional group changes very rapidly over the physiological pH range. At pH values below pH 7.5 the group has low reactivity, and at values above pH 7.5 it has high reactivity. The intermediate reactivity values, which typically occur around the pK_a of a functional group, are not observed. At the lower pH values the reactivity–pH profile approximates the general shape expected for a titration curve, whereas at higher pH values the data points are more scattered about the theoretical curve. These data indicate that the microenvironment of the B-chain phenylalanine *N*-terminus in the free monomeric form of insulin is changing over the physiological pH range and that this change is concomitant with the

deprotonation of the α-amino function. At higher concentrations of insulin, this α-amino group gives a more typical reactivity–pH profile. The pK_a value determined in these studies is similar to that obtained in the present study. At concentrations of insulin where the dimer associates to form higher oligomers, the phenylalanine *N*-terminus was found to be significantly more reactive on the basis of a Brönsted plot. However, this group was found to have a reactivity in the free monomer that is much closer to that expected for a primary amine with the same pK_a value (Fig. 5 and Table 1). Both the lowered reactivity and unusual reactivity–pH profile observed for the phenylalanine-B1 residue in the present study compared with studies at higher concentrations indicate that the microenvironment of the B-chain *N*-terminus of insulin is different in the free monomer of insulin and in insulin dimers and higher oligomers. The different properties between the associated and free states are consistent with the X-ray-crystallographic structure, which shows that this residue lies on an extremity of the monomeric unit and is involved in monomer–monomer interactions in the hexamer. However, the present data support the previous conclusion (Kaplan *et al.*, 1984) that the environment of this residue changes only on dissociation of the dimer to free monomer. This observation does not support the prediction (Blundell *et al.*, 1972) that, in solution, the dimeric form of insulin involves association at the face opposite to the phenylalanine residue.

The reactivity–pH profile for the histidine residues in the free insulin monomer is indicative of two ionizations of the histidine residues: one in approximately the physiological pH range and a second at a much higher pH. This second ionization is, indeed, expected for all imidazole functions. In the previous study (Chan *et al.*, 1981) this second ionization was not observed, since at pH values above pH 8.0 the reactivity of the imidazole functions of the histidine residues decreased. This reactivity, however, was taken relative to the internal standard imidazolyl-lactic acid, which itself has a second ionization at higher pH. These results can be interpreted as showing that the second ionization of the imidazole function results in a greater increase in the reactivity of imidazolyl-lactic acid than in the average reactivity of the two histidine residues. In the present study alanylalanine was used as an internal standard. Since this reactive moiety has an α-amino function with one ionization, the second ionization of the histidine imidazole function is readily observed. Another possible explanation is that the two histidine residues have widely different pK_a values, one with a pK_a value of 6.8 and the other with a pK_a greater than 10.

On the basis of the X-ray-crystallographic data an intramonomer hydrogen bond involving the δ-nitrogen atom of the imidazole ring to the A7-residue carbonyl group is postulated. In the free monomer, the fact that the average reactivity of the histidine residues is approx. 6 times that of imidazolyl-lactic acid indicates that some localized structure exists in the vicinity of at least one of the two histidine residues. The decreased reactivity relative to that seen in higher oligomers (Table 1) may indicate a slightly different conformation in this region of the monomeric unit in which the hydrogen bond involving the δ-nitrogen atom, is less directed.

The reactivity–pH profiles for the functional groups in the free insulin monomer support the conclusion reached in the concentration-dependence study (Kaplan *et al.*, 1984), namely that the conformation of the insulin monomeric unit in its free form does not differ substantially from that in its associated forms. The observation that des-(pentapeptide)-insulin has a very similar X-ray-crystallographic structure to insulin (Ru-Chang *et al.*, 1983), but is monomeric in solution, is in agreement with this conclusion. Thus use of the X-ray-crystallographic structure of insulin hexamers as a model for the structure of the free monomeric unit in very dilute solution appears justified.

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