Chemical properties of the functional groups of insulin

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The method of competitive labelling [Kaplan, Stevenson & Hartley (1971) *Biochem. J.* **124**, 289–299] with 1-fluoro-2,4-dinitrobenzene as the labelling reagent [Duggleby & Kaplan (1975) *Biochemistry* **14**, 5168–5175] was used to determine the chemical properties, namely pK and reactivity, of the amino groups, the histidine residues and the tyrosine residues of the dimeric form of pig zinc-free insulin at 20.0°C. The *N*-terminal glycine residue of the A-chain has a pK of 7.7 and a slightly higher than normal reactivity. The *N*-terminal phenylalanine residue of the B-chain has a pK of 6.9 and is approximately an order of magnitude more reactive than a corresponding amino group with the same pK value. The lysine ϵ -amino group has an unusually low pK of 7.0 but has approximately the expected reactivity of such a group. In the case of the two histidine and four tyrosine residues only the average properties of each class were determined. The histidine residues have a pK value of approx. 6.6, but, however, their reactivity is at least an order of magnitude greater than that of a free imidazole group. The tyrosine residues have a pK value of approx. 10, but their average reactivities are substantially less than for a free phenolic group. At alkaline pH values above 8 the reactivity of all the functional groups show sharp discontinuities, indicating that insulin is undergoing a structural change that alters the properties of these groups.

The precise structural requirements for the binding of the insulin molecule to its receptor are not clear at present. X-ray-crystallographic and chemical-modification studies, summarized by Blundell *et al.* (1972), have shown that various regions of the insulin molecule are involved in the recognition process by the receptor and that the integrity of the tertiary structure is essential for activity. However, an aspect of the structure–function relationships in the insulin molecule that has received very little attention is the ionization state of the functional groups in the active form of insulin. It has been shown that the binding of insulin to plasma membranes is pH-dependent (Kahn, 1975; Goldfine, 1978), which implies that the state of ionization of groups on the insulin molecule and/or receptor is a crucial structural feature.

In a previous study (Sheffer & Kaplan, 1979) performed at 10°C and 37°C with the competitive labelling technique (Kaplan *et al.*, 1971), with [3H]acetic anhydride as the labelling reagent and phenylalanine as the standard reference nucleophile, it was found that the amino groups of insulin had

unusual chemical properties. The *N*-terminal phenylalanine residue had a pK of 7.2, which is somewhat lower than the 8–8.5 range normally expected (Cohn & Edsall, 1943) for the *N*-terminus of a polypeptide. An even larger lowering of the expected pK value was observed for the lysine ϵ -amino group, which appeared to have a pK of 7.8 at 10°C. The *N*-terminal glycine residue, with an observed pK of 7.9, appeared to have more normal properties. However, both the *N*-terminal glycine residue and the lysine ϵ -amino group, but not the *N*-terminal phenylalanine residue, had sharp discontinuities in their pH–reactivity profiles at pH 8. It could be argued that the unusual properties observed for these groups result from some specific interaction between insulin and the labelling reagent (acetic anhydride) or the internal standard (phenylalanine). Although there is no evidence to support this possibility, the properties of the groups appeared to be so unusual as to warrant further investigation.

The major objective of the present study is to determine whether the unusual properties observed for the amino groups of insulin are a function of the reagents employed or an inherent property of the insulin molecule. This study was performed with [3H]Dnp-F as the labelling reagent and imidazolyl-

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

lactate as the standard nucleophile. Advantage is taken of the fact that insulin contains three unique amino groups of glycine-A1, phenylalanine-B1 and glycine-B29, so that the competitive-labelling procedure described by Duggleby & Kaplan (1975), in which the Dnp-amino acids are isolated after acid hydrolysis, could be employed. If the compounds used do not have any specific interactions with insulin, then the chemical properties observed with acetic anhydride and Dnp-F should be very similar.

Experimental

Materials

Pig zinc insulin (Connaught Laboratories, Toronto, Ont., Canada) was converted into the zinc-free form as previously described (Sheffer & Kaplan, 1979). [3,5-³H]- and [U-¹⁴C]-Dnp-F were from Amersham/Searle (Don Mills, Ont., Canada), and unlabelled Dnp-F was from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Porapak Q was supplied by Waters Associates (Milford, MA, U.S.A.). Other chemicals were high-purity preparations from commercial sources. Acetyl-*im*-Dnp-histidine and Dnp-imidazolyl-lactic acid were prepared as described by Cruickshank *et al.* (1971) and Duggleby & Kaplan (1975).

Trace labelling

Pig zinc-free insulin was suspended at a concentration of 0.412 mg/ml in a buffer consisting of 5.0 mM-*N*-methylmorpholine, 5.0 mM-acetic acid, 5.0 mM-boric acid and 100 mM-KCl. The internal standard, L-β-imidazolyl-lactic acid, was added to a final concentration of 0.32 mM. For each labelling a portion (2.50 ml) of this solution was equilibrated at 20°C and adjusted to the desired pH value by the addition of 1 M-KOH. Acetonitrile (50 μl) containing 0.35 mCi of [³H]Dnp-F (30 Ci/mmol) was added with vigorous stirring. After 6 h the reaction was terminated by the addition of 1 drop of conc. HCl.

Dinitro[¹⁴C]phenylation

The trace-labelled insulin was denatured by the addition of urea to give a concentration of 8 M. Then NaHCO₃ (0.5 g) was added followed by 25 μl of [¹⁴C]Dnp-F (13.3 μCi) in 50% (v/v) acetonitrile. After 16 h at 25°C the reaction was terminated by lowering the pH to 2 with HCl. Unlabelled Dnp-insulin (2.5 mg), prepared by treating 250 mg of pig insulin with 250 μl of Dnp-F in 5 ml of 8 M-urea containing 0.5 g of NaHCO₃, was added as carrier to each sample. Addition of 1 drop of octanol before that of HCl considerably diminished foaming. Unchanged Dnp-F, 2,4-dinitrophenol and other side products were removed by extraction with diethyl ether. The above procedure results in precipitation of the Dnp-insulin, which was

separated from the soluble internal standard by centrifugation at 10000 g for 10 min. The Dnp-insulin was washed twice with 2 ml portions of 10 mM-HCl and then hydrolysed in 6 M-HCl *in vacuo* for 16 h at 110°C.

Purification of internal standard

The supernatant from the centrifugation contains the derivative of the internal standard, [³H,¹⁴C]Dnp-imidazolyl-lactic acid. Unlabelled carrier Dnp-imidazolyl-lactic acid (1 mg) was added to the supernatant before application to a column (7 mm × 30 mm) of Porapak [pre-equilibrated in 95% (v/v) ethanol for 3 h and then washed with 10 mM-HCl]. After a washing with 20 ml of 10 mM-HCl, the Dnp-imidazolyl-lactic acid was eluted with 80% (v/v) acetone. Further purification was performed by electrophoresis as previously described by Duggleby & Kaplan (1975).

Purification of α-N-Dnp-glycine and α-N-Dnp-phenylalanine

The hydrolysate of Dnp-insulin was extracted with several 2 ml portions of diethyl ether. After the ether extract had been passed through a silica-gel column to remove trace amounts of dinitrophenol (Steven, 1962), the Dnp-glycine and Dnp-phenylalanine were separated by t.l.c. (Kaplan *et al.*, 1978).

Purification of ε-Dnp-lysine, O-Dnp-tyrosine and im-Dnp-histidine

After ether extraction of the hydrolysate, the aqueous portion contains ε-Dnp-lysine, O-Dnp-tyrosine and *im*-Dnp-histidine. The aqueous phase was freeze-dried, and the Dnp components were separated by high-voltage electrophoresis for 1 h on Whatman 3MM paper at pH 6.5 and then at pH 2.1 with a voltage gradient of 60 V/cm.

Radioactivity measurements and calculations

The elution of the Dnp derivative from the chromatograms and electrophoretograms and subsequent scintillation counting of radioactivity were performed as previously described (Duggleby & Kaplan, 1975). The pH-dependent second-order velocity constants for the reaction of Dnp-F with the group of interest were calculated from the equation:

$$\alpha_x r = \alpha_s \frac{({}^3\text{H}_x/{}^{14}\text{C}_x)}{({}^3\text{H}_s/{}^{14}\text{C}_s)} \quad (1)$$

where α_x is the degree of ionization of the functional group under study, α_s is the degree of ionization of the imidazolyl-lactate (pK = 7.27; Cruickshank & Kaplan, 1975), *r* is the pH-independent second-order velocity constant for the reaction of the functional group under study relative to that for the internal

standard imidazolyl-lactate, and ($^3\text{H}_x/^{14}\text{C}_x$) and ($^3\text{H}_s/^{14}\text{C}_s$) are the corresponding $^3\text{H}/^{14}\text{C}$ radio-activity ratios for the group and internal standard respectively.

Competitive labelling of peptides and amino acids

Mixtures containing the following amino acids and peptides were prepared. Mixture I: Ala-Gly, Asn, Glu-Gly, Gly, Leu, Bz-Gly-Lys, Phe-Gly and *N*-acetyl-Tyr-NH₂. Mixture II: Ala, Gln, Gly, Leu, Bz-Gly-Lys, Phe and *N*-acetyl-Tyr-NH₂. Mixture III: (Ala)₅, (Gly)₆, Leu, Bz-Gly-Lys, (Phe-Gly)₂ and *N*-acetyl-Tyr-NH₂. A sample of each mixture was diluted to 2.50 ml, so that the final solution contained 500 nmol of each amino acid or peptide and was 100 mM with respect to KCl and 10 mM with respect to sodium borate. The final pH was adjusted to 9.50, and 25 μl of [^3H]Dnp-F (0.035 mCi, specific radioactivity 14.5 Ci/mmol) in acetonitrile was added for each labelling, which was carried out in duplicate. After reaction for 2 h at 20°C, 0.5 g of NaHCO₃ was added to each sample and followed by the addition of 25 μl of [^{14}C]Dnp-F (8.3 μCi) in 50% (v/v) acetonitrile. The reaction mixtures were shaken for 24 h at room temperature. After acidification to pH 2 with conc. HCl, the samples were passed over a Porapak Q column as described above. The eluates from the column containing the Dnp derivatives of the peptides and amino acids were dried under N₂ and then hydrolysed in 6M-HCl *in vacuo* at 110°C for 18 h. The Dnp-amino acids in the hydrolysate were purified as described for the Dnp-insulin hydrolysate.

Determination of rate constants for the reaction of amino acids and peptides with Dnp-F

The ionization constants of the amino groups of the amino acids and peptides were determined by direct titration of 250 nmol of each compound in 0.10M-KCl at 20°C with 0.20M-KOH. The radio-

activity measurements were performed as described for the Dnp-insulin hydrolysate, and the second-order rate constants were determined by using eqn. (1), where α_s was taken to be the degree of ionization of glycine in mixtures I and II and that of leucine in mixture III. The absolute rate of reaction of glycine with Dnp-F was determined by use of a pH-stat as described by Cruickshank & Kaplan (1975).

Results

Table 1 summarizes the results obtained for the reactivity of the functional groups of insulin. Fig. 1 shows a plot of $\alpha_x r$ versus pH for the *N*-terminal glycine and phenylalanine residues of insulin. The experimental points closely follow titration curves with a p*K* of 7.7 for the *N*-terminal glycine residue and a p*K* of 6.9 for the *N*-terminal phenylalanine residue with pH-independent second-order velocity constants of 25 and 32 relative to imidazolyl-lactic acid. Given a pH-independent rate constant of 0.0443 M⁻¹·min⁻¹ (Cruickshank & Kaplan, 1975), the velocity constant for the reaction of the *N*-terminal glycine residue with Dnp-F is 1.08 M⁻¹·min⁻¹, and 1.42 M⁻¹·min⁻¹ for the *N*-terminal phenylalanine residue.

Fig. 2(b) shows a plot of the results for the ϵ -amino group of lysine-B29. It is clear that a single titration curve cannot be fitted to the experimental points obtained over the entire pH range studied. The best approximation to a titration curve, p*K* 7.0 and a second-order velocity constant of 8.2 relative to imidazolyl-lactate or 0.36 M⁻¹·min⁻¹ in absolute units, is obtained when only the results obtained below pH 8 are considered. The linear plot of the data (Fig. 2a) clearly shows the discontinuity that occurs in the reactivity above pH 8. The experimental point at pH 6 appears to be aberrant; however, at the extreme acid end of such a titration curve a

Table 1. Reactivity of glycine-A1, phenylalanine-B1, lysine-B29, histidine-(B5 + B10) and tyrosine-(A14 + A19 + B16 + B26) residues of insulin
For details see the text.

pH	α_s	$^3\text{H}_s/^{14}\text{C}_s$	$^3\text{H}_x/^{14}\text{C}_x$					$\alpha_x r$				
			Gly	Phe	Lys	His	Tyr	Gly	Phe	Lys	His	Tyr
5.53	0.0179	0.642	0.787	31.12	9.89	18.25	2.16	0.022	0.869	0.276	0.509	0.060
6.01	0.0521	1.55	2.400	32.86	8.28	24.99	2.95	0.08	1.107	0.278	0.838	0.099
6.38	0.1165	0.798	9.83	55.81	10.62	52.68	3.53	1.43	8.15	1.55	7.69	0.516
7.00	0.3494	1.62	14.45	93.33	19.00	80.90	7.95	3.11	20.10	4.09	17.43	1.71
7.51	0.6347	2.73	35.23	113.57	25.64	86.41	10.80	8.18	26.88	5.96	20.07	2.51
8.00	0.8430	4.12	65.04	122.74	41.19	79.60	24.52	13.30	23.15	9.42	16.28	5.01
8.48	0.9421	4.47	93.36	151.23	69.82	62.40	55.76	19.68	31.88	14.71	13.15	11.75
8.99	0.9813	4.48	110.23	131.67	53.86	53.30	89.18	24.15	28.84	11.80	11.68	19.53
9.53	0.9945	4.04	120.60	129.34	129.17	46.30	107.92	29.70	31.86	31.82	11.33	26.59
10.10	1.0000	4.92	102.20	94.74	137.45	44.15	121.77	20.76	19.24	27.91	8.97	24.74

Fig. 1. Effect of pH on the reactivity of the N-terminal glycine residue (▲) and the N-terminal phenylalanine residue (●) of insulin

For details see the text. The continuous lines show how theoretical titration curves with a pK of 7.7 and an r of 25 and with a pK of 6.9 and an r of 32 fit the experimental points for the N-terminal glycine and phenylalanine residues respectively.

Fig. 2. Effect of pH on the reactivity of the lysine ϵ -amino group of insulin

For details see the text. The continuous line in (b) is a theoretical titration curve with a pK of 7.0 and an r of 8.2. (a) shows a linear plot of the experimental points with the continuous straight line corresponding to the theoretical titration curve in (b).

relatively small error is greatly magnified in the linear plot.

Fig. 3 is a plot of the results for the average reactivity of the two histidine and four tyrosine residues. The histidine residues have an average pK value of approx. 6.5 with an average reactivity 20 times that of imidazolyl-lactic acid, and the four tyrosine residues have an average pK value of approx. 10 with a reactivity about 50 times that of imidazolyl-lactic acid. The two histidine residues are 'super-reactive' compared with reactivity relative to imidazolyl-lactate ($\alpha_x r$ in Table 1) by at least an order of magnitude over normal imidazole groups (Cruikshank & Kaplan, 1975), whereas the four tyrosine residues (average $k = 2.2 \text{ M}^{-1} \cdot \text{min}^{-1}$, assuming an average pK of 10.0) appear to have a lower than normal reactivity when compared with the reactivity of the phenolic hydroxy group of *N*-acetyltyrosine amide ($k = 29 \text{ M}^{-1} \cdot \text{min}^{-1}$).

Fig. 4 is a Brønsted plot (Brønsted & Peterson, 1924) of the reaction of a series of standard amines with Dnp-F. The reactivity of these amines towards Dnp-F parallels their basicity and follows the Brønsted equation:

$$\log k = \alpha + \beta \cdot \text{pK}$$

Fig. 3. Effect of pH on the reactivity of the imidazole side chains of histidine residues (●) and the phenolic side chains of tyrosine residues (▲) of insulin

For details see the text.

Table 2. *Chemical properties of insulin with different competitive labelling reagents*
For details see the text. Abbreviation: RR, relative reactivity (see the text).

Amino group	Chemical property	Competitive labelling reagents and internal standards	
		Acetic anhydride (10°C)*, phenylalanine	Dnp-F (20°C), imidazolyl-lactate
Glycine	pK	7.9	7.7
	RR	2.6	2.5
Phenylalanine	pK	7.2	6.9
	RR	6.6	7.5
Lysine	pK	7.8	7.0
	RR	0.23	1.7

* Sheffer & Kaplan (1979).

Fig. 4. *Brønsted plot for a series of primary amino compounds (▲) and reactive amino groups of insulin (◻)*
For details see the text. The continuous line is a least-squares fit to the data. $\log k = (0.455 \pm 0.045) pK - (3.86 \pm 0.40)$.

where $\alpha = -3.86 \pm 0.40$ and $\beta = 0.455 \pm 0.045$. The reactivities of the amino groups of insulin are shown in relation to this plot, and it is clear that the *N*-terminal phenylalanine residue is approximately an order of magnitude more reactive than would be expected on the basis of the Brønsted plot. The *N*-terminal glycine residue and ϵ -amino group of lysine lie relatively close to the curve and therefore appear to have 'normal' properties.

Discussion

Many chemical modifications have been performed on the functional groups of insulin with a view to elucidating their role in the insulin molecule (summarized by Blundell *et al.*, 1972). It is evident from these studies that the functional groups, in

particular the *N*-termini and the ϵ -amino groups of lysine-B29, are readily available for reaction. The X-ray crystallographic structure is in general agreement with these findings. However, the ionization states of the functional groups in the active form of insulin or the dynamic properties of the insulin molecule are not obvious from this structure. The present study confirms the observations made by Sheffer & Kaplan (1979) that the amino groups of insulin have rather unusual and unexpected properties. These properties undoubtedly reflect their particular structural or functional role within the molecule as well as the dynamic properties of the molecule.

Under the experimental conditions employed it would appear that insulin exists primarily as the dimer (Jeffrey *et al.*, 1976; Bradbury & Brown, 1977). Independent observations in our laboratory made with Sephadex G-75 (superfine grade) gel filtration have confirmed this in the pH range 6–7.5. Above pH 8, there are obvious discontinuities in the pH–reactivity profiles that probably result from a pH-dependent structural alteration with a resultant change in the environment of the functional groups. Below pH 8 the experimental points can be fitted to theoretical titration curves, which indicates that the environment of these groups remains constant in this pH range.

An attempt to answer the question as to whether the unusual chemical properties observed are the inherent properties of the insulin molecule or a function of the reagents can be made by comparing the parameters in Table 2 for the reactions with acetic anhydride and Dnp-F. Clearly, the chemical properties (namely pK and relative reactivity) of each *N*-terminus are very similar towards both reagents. Since these amino groups will very probably have positive ΔH values of approx. $41.8 \text{ kJ} \cdot \text{mol}^{-1}$ (Cohn & Edsall, 1943), the pK values obtained at 10°C and 20°C would, within experimental error, be identical. Their deviations from Brønsted plots for primary amines as indicated by their relative reactivity (RR), i.e. the reactivity

relative to an amine of the same pK but that lies on a Brønsted plot (Kaplan *et al.*, 1971), again are very similar (Table 2). The *N*-terminal phenylalanine residue is clearly 'super-reactive', whereas the *N*-terminal glycine residue appears to have 'normal' properties towards both reagents. On the other hand, the lysine ϵ -amino group appears to exhibit a different pK value towards the two reagents. This group, however, is so unusual in its properties that the difference could be accounted for by the effect of temperature alone. The most important consideration is that its pK value is displaced by at least 2 pK units from what might be considered a normal pK for a lysine ϵ -amino group. Therefore, since similar properties were observed with labelling reagents and standard nucleophiles differing considerably in their physicochemical properties, the results strongly indicate that these particular compounds do not have any specific binding interaction with insulin and that the unusual properties of the amino groups are inherent to the insulin molecule.

The procedure used in the present study to quantify the reactivity involves the isolation of Dnp-derivatives after acid hydrolysis. As the three amino groups are unique, an unequivocal assignment of parameters can be made to these groups, i.e. the Dnp-glycine, Dnp-phenylalanine and ϵ -Dnp-lysine can arise from only one position in the primary structure (Blundell *et al.*, 1972). With the tyrosine and histidine groups, of which there are four and two respectively, these Dnp derivatives will arise from more than one position and thus an unequivocal assignment of parameters cannot be made to individual groups, but the average for the particular class can be obtained.

The histidine and tyrosine residues have pK values in the expected range for such groups, but clearly at least one histidine residue is 'super-reactive'. The X-ray crystallographic structure shows that histidine-B5 is in a position to form a hydrogen bond with the carbonyl group of residue A7. This would account for the high reactivity of this histidine residue in the same manner as the active-centre histidine residue of chymotrypsin (Cruickshank & Kaplan, 1975). It is not possible to infer much from the average reactivity of the four tyrosine residues, except to say that on the basis of the reactivity of *N*-acetyltyrosine amide at least some of the tyrosine residues are buried.

It should be emphasized that there is no reason *a priori* why any functional group in a protein should fit a titration curve whatever the physical approach used to determine this curve. There are obvious discontinuities in our reactivity data for all three amino groups. This indicates only that the insulin molecule is a dynamic structure whose properties are changing with pH and that hence the environments of the functional groups are

changing. The titration curves are used primarily as a theoretical framework within which the experimental results are to be analysed. Between pH values 5.8 and 8 the experimental points fit theoretical titration curves, which means that the micro-environment of the groups is constant in this range. The discontinuities above pH 8 indicate that some alteration in the structure is occurring. Similar discontinuities were observed with acetic anhydride as the labelling reagent (Sheffer & Kaplan, 1979).

It is not evident from the X-ray crystallographic structure (Blundell *et al.*, 1972) exactly what structural features would give rise to the unusual chemical properties of the lysine ϵ -amino group. The low pK value could be accounted for by its proximity to the protonated glycine α -amino group, but the position of the ϵ -amino group could not be precisely defined, owing to apparent local disorder in this region. This, however, does appear to be the most likely explanation for the observed low pK value. The discontinuity in the reactivity data at pH 8 could result from deprotonation of the glycine amino group, at which point the pK of the lysine ϵ -amino group would be expected to return to more normal values.

On the basis of the X-ray crystallographic structure, it might be expected that the *N*-terminal glycine residue would have abnormal properties, since it is in close proximity to the residue-A4 carboxylate group, tyrosine-A19 and lysine-B29 ϵ -amino group. Interaction with any of these groups would be expected to confer unusual properties on this *N*-terminus. One can only speculate that the net effect of any interactions is such as to be mutually compensating, e.g. the effect of the positive charge on the lysine residue compensating for the effect of the negative charge on the residue-A4 carboxylate group.

The reason for the low pK value for the *N*-terminal phenylalanine residue is again not clear. The speculation by Blundell *et al.* (1972), that this α -amino group may form a salt bridge with a carboxylate group, is not supported by our data. It is more likely that the deprotonated form of this group is involved in hydrogen-bonding with the carboxylate group, as this would account for its low pK as well as its high reactivity. It is also possible that this group is influenced by the positive end of a helix dipole, which also would tend to lower its pK value.

Bradbury & Brown (1977) have studied the dimethylamino derivative of the amino groups of insulin by ^{13}C n.m.r. They obtained pK values of 6.7 and 8.0 at 25°C for the α -amino groups of insulin, and tentatively assigned these to the *N*-terminal phenylalanine and glycine residues respectively. Our results support their assignment, as well as the apparent pK values obtained. However, their value

of 11.2 obtained for the lysine ϵ -amino group differs so drastically from the value of 7.0 obtained in the present study that the only conclusion that can be reached is that different forms of insulin must be involved. Unfortunately, they apparently did not obtain any results for the lysine ϵ -amino group below pH 7.5, and the pK value reported was obtained at high pH. Under these conditions insulin is probably in monomeric form, whereas our data apply to the dimeric form. It is possible that the pK value of the lysine ϵ -amino group differs in the two forms of insulin; however, in our experience with competitive labelling at high pH values (Kaplan *et al.*, 1971; Kaplan, 1972; Sheffer & Kaplan, 1979), proteins invariably undergo structural changes, and thus their pK value may correspond to a denatured form of insulin.

In summary, the present study of insulin shows that at physiological pH values the *N*-terminal phenylalanine-B1 and the ϵ -amino group of lysine-B29 are deprotonated and the *N*-terminal glycine residue is partially deprotonated. These amino groups are therefore in a very reactive state under physiological conditions, and should react with any electrophilic metabolites present. It is tempting to speculate that, because of the unusual properties of the amino groups in dimeric insulin, they may have more than a passive structural role in the molecule and play a dynamic functional role in the mechanism of action of insulin.

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