Differential ultraviolet spectra of insulin*, **, ***

The ultraviolet absorption spectra of proteins are usually shifted towards longer wavelengths when compared with the corresponding spectra of mixtures of their component amino acids[1]. Above 260 mμ, where the ultraviolet spectrum of proteins arises essentially from their aromatic residues[1], this shift is attributable to two effects:

(a) the involvement of the aromatic amino acids in peptide bonds (vicinal effect), and

(b) the difference in environment of the aromatic R group in the protein and in the free amino acid.

In the case of tyrosine the second effect can arise if tyrosyl residues are hydrogen bonded. The involvement of the phenolic hydroxyl group in a hydrogen bond should increase the contribution of the quinoid form and shift the spectrum to longer wavelengths, as observed when the –OH group is ionized[1]. NAGAKURA AND BABA found such a shift in hydrogen bonded phenols[2], and the suggestions that this effect occurs in proteins have been frequent (see [1] for further references).

In order to investigate this shift and its implications for the internal structure of proteins, we have used the technique of *differential* spectrophotometry, and have applied it to beef zinc-free insulin and several other proteins. Insulin contains four tyrosyl residues in a molecular weight unit of 6000. One of these (the 26th residue of the B chain) is liberated from the parent molecule as part of a heptapeptide by the action of trypsin[3,4] (see Fig. 1). If this tyrosyl residue were hydrogen bonded to an acceptor on the remaining portion of the insulin molecule, then tryptic hydrolysis should break the hydrogen bond and shift the ultraviolet spectrum to shorter wavelengths. This is indeed observed. Fig. 2 illustrates the differential spectrum obtained (using either a Beckman DU or DK ultraviolet spectrophotometer) when native insulin at pH 7 is compared with its tryptic digest at the same pH§. The differential spectrum shown in Fig. 2 arises from the spectral shift.

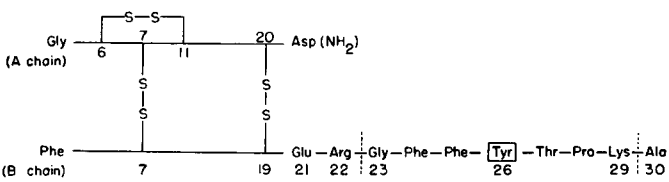

Fig. 1. Schematic representation of the SANGER structure[5] of insulin. The two bonds split by trypsin[3,4] are indicated by vertical dashed lines.

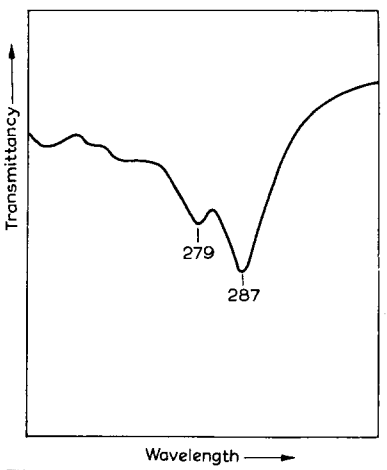

Fig. 2. Differential ultraviolet spectrum obtained when native insulin is compared to a tryptic digest of insulin at pH 7 (concentration, 0.025%).

Since the maximum difference between these two samples was found at 287 mμ the shift was not due to a small difference in degree of ionization of tyrosine in the two samples. In the latter case the maximum would be observed at 294 mμ[1]. The differential spectrum was further investigated at 287 mμ as a function of time of tryptic digestion. The results, expressed as difference in optical density, are shown in Fig. 3 and are in agreement with the assumption that the spectral shift accompanies the tryptic hydrolysis of the arginyl-glycyl bond (alanine being liberated much more rapidly[4]). The shift is almost certainly not due to the vicinal effect since the tyrosyl residue occupies a central position in the heptapeptide and thus is insulated by three residues from both peptide bonds broken. The shift thus appears to be due to the rupture of a tyrosyl hydrogen bond as proteolysis occurs.

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§ In order to make the overall composition of both solutions identical for spectrophotometric observations soybean trypsin inhibitor and trypsin were added to the native insulin, and the inhibitor to the tryptic digest.

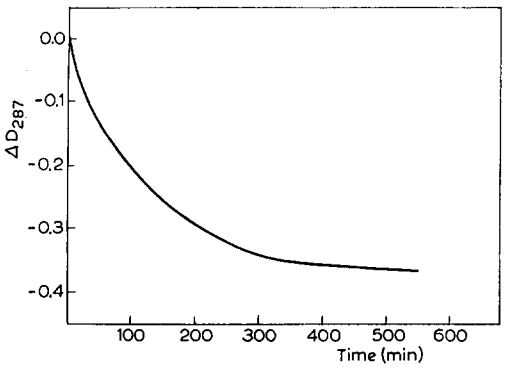

Fig. 3. Decrease in optical density at 287 mμ of an insulin solution as a function of time of tryptic digestion. Insulin, 0.50%; Trypsin, 0.01%

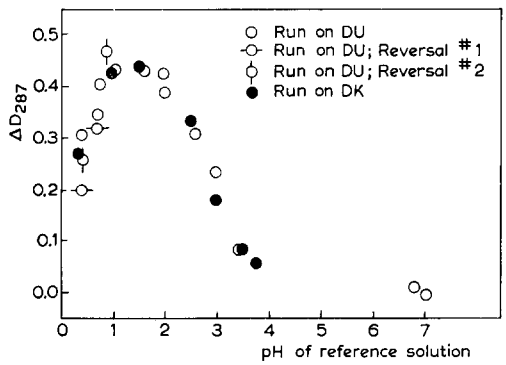

Fig. 4. Difference in optical density at 287 mμ between native insulin at pH 7 and native insulin at the pH indicated. No points could be obtained between pH 4 and 6.5 because of the insolubility of insulin in this pH range.

Since it was considered likely that a carboxylate ion is the acceptor for the tyrosyl proton in the hydrogen bond, the differential spectrum of *native* insulin was obtained as a function of pH. Insulin at pH 7, when compared with insulin at pH 2, gave a curve almost identical with that of Fig. 2, when corrected for the difference in optical density of the solvents, thus suggesting that in *native insulin* the hydrogen bond present at pH 7 was broken at pH 2. The full titration curve was obtained from the difference in optical density at 287 mμ between a solution of native insulin at pH 7 and a similar one at any other pH used as a reference. The titration curve is reversible throughout the pH range and is shown in Fig. 4. The drop in ΔD_{287} as the pH is lowered from 1 to 0.5 is as yet unexplained. From a consideration of the region at and above pH 1 it is seen that the pK of the acceptor group is approximately 3. Since this pK should be lower than the intrinsic pK° of the acceptor group due to hydrogen bonding[6] and to electrostatic effects on the acid side of the isoelectric point (pI = 5.60[7]), the observed value can be taken as an indication that the acceptor group is either a C-terminal carboxyl (pK° = 3.6[7]) or a side chain carboxyl group of a glutamyl or aspartyl residue (pK° = 4.7[7]). We can probably rule out the B 30 alanine since this amino acid is liberated faster[4] than the tyrosyl···carboxylate ion bond is ruptured (see Fig. 3).

It is thus concluded that native insulin contains a hydrogen bond between the B 26 tyrosyl residue and an as yet unidentified carboxylate ion acceptor group. This hydrogen bond can be broken either by neutralizing the carboxylate ion with acid or by removing the heptapeptide by tryptic hydrolysis.

The method of differential ultraviolet spectrophotometry appears to be of general applicability in the detection and *location* of tyrosyl hydrogen bonds in proteins. Preliminary work on the differential spectrum of lysozyme[8] has shown that at least two of its three tyrosyl residues are hydrogen bonded to ionizable groups. This technique is also being applied to the polymerization of monomeric fibrin which may involve the formation of an intermolecular hydrogen bond with tyrosyl as a donor.

Further work is in progress at present on improvements in the technique of differential ultraviolet spectrophotometry, on the location of the carboxylate ion involved with the B 26 tyrosyl group in a hydrogen bond in insulin, and on the applicability of BEER's law.

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