A Reaction of Glucose with Peptides

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Valylhistidine (Val-His) reacts with glucose (Glc) in a mixture of pyridine and acetic acid to form glucosylvalylhistidine (Glc-Val-His). The pK of the α -amino group is thereby lowered to about 5.6 as judged by electrophoretic mobility. The reaction:

also occurs in an aqueous solution of pyridine and acetic acid of pH 6.2 at 50°C, in which it exhibits a half-time of about 30 h and a dissociation constant of about 0.3 M. Isoleucyltyrosine and glucose react similarly in aqueous solution. The Glc-Val-His has the chromatographic, electrophoretic and chemical properties reported by Holmquist & Schroeder (1966a) for the substance released by proteolysis from the *N*-terminus of the β -chains of haemoglobin A_{1c}; the value of the dissociation constant means that the concentration of haemoglobin A_{1c} found naturally could be explained by reaction of haemoglobin A with the blood glucose.

Holmquist & Schroeder (1966a) showed that the *N*-terminus of the β -chains of haemoglobin A_{1c} were blocked with an alkali-labile group which diminished its pK . The group ceased to be removable by alkali, or by acid hydrolysis, after reduction with borohydride. They suggested that these properties could be explained by the presence of an imine, although this seemed strange in view of the ease of hydrolysis of imines derived from aliphatic amines (e.g. Cordes & Jencks, 1963), which Holmquist & Schroeder (1966a) noted. Bookchin & Gallop (1968) characterized the terminal peptide of borohydride-reduced haemoglobin A_{1c} as possessing a $\text{CH}_2\text{OH}-[\text{CHOH}]_4-\text{CH}_2-$ group on the α -amino group of the terminal valine. This suggested a possible reconciliation of the theory of Holmquist & Schroeder (1966a) with the known instability of imines. The imines of sugars (I in Scheme 1) are known to equilibrate rapidly with the glycosylamine form (II) (Isbell & Frush, 1958; see also Capon, 1969, pp. 448–450), presumably because attack by the 5-hydroxyl group of the sugar is much faster than by

water. This would greatly increase the stability of the complex, since there might be very little in the unstable form (I), although enough to explain the observed reaction of the complex with borohydride to form the characterized product (III). The present paper describes a study of glucosylvalylhistidine. Its properties prove to be consistent with formulation of its structure as $\text{I} \rightleftharpoons \text{II}$, and also with its being the material isolated from haemoglobin A_{1c} by Holmquist & Schroeder (1966a).

Materials and Methods

Materials

Valylhistidine was made by the general procedure for peptide synthesis of Belleau & Malek (1968), as follows. Histidine methyl ester dihydrochloride (5 mmol), *N*-*t*-butyloxycarbonylvaline (5 mmol) and triethylamine (10 mmol) were suspended in 50 ml of ethyl acetate and stirred overnight with 6 mmol of 2-ethoxy-*N*-ethoxycarbonyl-1,2-dihydroquinoline at

Scheme 1. *Interconversions of sugar derivatives*
 For details see the text.

room temperature. The solution was extracted with water (20ml) and with a buffer of pH 10 ($2 \times 20\text{ml}$; 0.4M-NaHCO_3 – $0.4\text{M-Na}_2\text{CO}_3$) to remove unreacted components. The ethyl acetate layer was filtered through silicone-treated paper (Whatman 1PS) and evaporated to dryness on a rotary evaporator. Most of the quinoline formed was removed in a desiccator evacuated to about 7 N/m^2 . The solid was dissolved in 7.5ml of trifluoroacetic acid to remove the butyloxy-carbonyl group and after 2h was again evaporated to dryness. The syrup was dissolved in water, adjusted to pH 6 with aq. NaOH, and extracted with diethyl ether to remove residual quinoline. It was then maintained at pH 12 by addition of 4M-NaOH in a pH-stat until the uptake of alkali was complete. The solution was extracted twice with ethyl acetate, diluted to 100ml with water, and passed through a column of the free acid form of a sulphonic resin (Zeo-Karb 225 SRC 14). The peptide was eluted from this with 1M-NH_3 and the solution was evaporated to dryness on a rotary evaporator. On dissolving the dry material in methanol it crystallized (yield 0.94g , 74%).

The histidine methyl ester dihydrochloride was made from histidine, methanol and thionyl chloride (Brenner & Huber, 1953), by using excess of the last, and cooling only to 0°C while it was added. The *N*-t-butyloxycarbonylvaline was made from valine and t-butylazidoformate (Schnabel, 1967); this in turn was made from t-butylcarbazate by the method of Carpino *et al.* (1964) but was not distilled.

Isoleucyltyrosine was made by similar methods, and finally was crystallized from water by adding acetone.

Glucosylvalylhistidine was made from finely ground glucose (0.25g , 1.41mmol) and valylhistidine (0.25g , 0.99mmol) which were stirred together in a mixture of 2ml of acetic acid and 2ml of pyridine at room temperature for 3 days. After 2 days paper electrophoresis showed that little valylhistidine remained. Most of the pyridine and acetic acid were removed by rotary evaporation, addition of water, and repeated evaporation. The syrup was dissolved in water and applied to a column ($25\text{cm} \times 1\text{cm}$) of a sulphonic resin (Zeo-Karb 225 SRC 16) equilibrated and developed with 0.3M -acetic acid– 0.3M -pyridine buffer at $60\text{ml} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$. The effluent containing the first histidine-positive peak (2 – 4.5 column volumes) was pooled and evaporated to dryness. The syrup was dissolved in methanol, and Glc-Val-His was precipitated by adding ether (yield 0.30g , 71%) (Found: C, 48.1 ; H, 6.8 ; N, 12.5 . $\text{C}_{17}\text{H}_{28}\text{N}_4\text{O}_8$ requires C, 49.0 ; H, 6.7 ; N, 13.5%).

Methods

Electrophoresis on paper was done as described by Ambler (1963); the paper being cooled by immersion in white spirit for electrophoresis at pH 3.5, and in

toluene containing 8% of pyridine for electrophoresis at pH 6.5. Material was detected on paper by reaction with diazotized sulphanilic acid (Pauly, 1904; Bailey, 1967) for histidine, or with ninhydrin. Reducing sugar was detected by dipping the paper in a solution of 0.1M -phthalic acid and 0.1M -aniline in acetone and heating.

Histidine-containing material in column effluents (0.3M -acetic acid– 0.3M -pyridine) was determined by reaction with diazotized sulphanilic acid, stabilizing the colour with ethanol (Sluyterman, 1960). The reagent was made by mixing 1vol. of 5% (w/v) NaNO_2 and 1vol. of 1% sulphanilic acid in 1M-HCl ; after 2min 2vol. of 15% (w/v) Na_2CO_3 were added, and the solution was used within 10min . Each sample of 1ml of effluent was mixed with 1ml of reagent; 2min later 3ml of 50% (v/v) ethanol was added and the E_{530} was read. It increased linearly with increasing amounts of valylhistidine up to the highest concentrations obtained from the columns.

Results

Preliminary characterization of Glc-Val-His

Valylhistidine, Glc-Val-His and borohydride-treated valylhistidine all gave valine and histidine on acid hydrolysis; after borohydride treatment, however, Glc-Val-His gave histidine but no valine. Spots of Glc-Val-His on paper gave positive reactions for histidine (Pauly reagent) and slowly for reducing sugar and with ninhydrin.

Electrophoretic characterization of Glc-Val-His

Table 1 shows the electrophoretic mobility of valylhistidine and of Glc-Val-His in the system usually described as having a pH of 6.5 [the paper was wetted with a buffer of 10% (v/v) pyridine and 0.4% (v/v) acetic acid, and immersed in toluene containing 8% (v/v) of pyridine]. In fact extraction of pyridine from the aqueous buffer in the paper by the coolant diminishes the pH, and addition of enough pyridine to the toluene to prevent this leads to dehydration of the paper (R. Fields, unpublished work). Therefore an estimate of the pH of the solution actually in the paper was obtained from the mobility of histidine, taking its pK as 6.0 (Sillén & Martell, 1964) and taking the mobility to be proportional to charge and to $(\text{molecular weight})^{-2/3}$ (Offord, 1966). Hence from the data in Table 1, the pH must have been 5.85 to give histidine a charge of 0.58 . The valylhistidine appears to have a charge of 0.87 , so that if its terminal amino and carboxyl groups are, as usual, fully charged, its histidine residue must have a pK of 6.7 . This high value may reflect the proximity of the charged terminal carboxyl group. If the further assumption be made that this histidine residue carries

Table 1. *Mobilities of peptides at pH '6.5'*

Electrophoresis was performed at 100V/cm for 20min. The apparent charge was obtained by multiplying the mobility by (molecular weight)^{2/3} and dividing by the corresponding function for lysine or aspartic acid. In five determinations the values for valylhistidine were 0.868 ± 0.011 and for Glc-Val-His 0.220 ± 0.010 (means $\pm$ s.e.m.). Square brackets denote calibration values.

Substance	Distance from origin (cm)	Corrected distance migrated (cm)	Mobility as % of lysine or aspartic acid	Apparent charge
Positive				
Neutral amino acids and Ile-Tyr	1.4	[0]	[0]	
Lys	16.6	15.2	[100]	[1]
Arg	15.7	14.3	94	1.04
His	10.0	8.6	57	0.58
Val-His	10.7	9.3	61	0.87
Glc-Val-His	3.0	1.6	11	0.22
Reduced Glc-Val-His	6.5	5.1	34	0.68
Negative				
Asp	-17.4	-18.8	[100]	[1]
Glu	-15.8	-17.2	92	1.00
Glc-Ile-Tyr	-3.1	-4.5	24	0.50

the same charge in Glc-Val-His, then the overall charge of 0.22 would be explained by a charge of +0.35 on the glucosylated nitrogen, and hence a pK for this group of 5.6. These values must, of course, be treated with caution, because Offord (1966) showed that peptides do not obey the Offord (1966) relationship very precisely. The pK value for the glucosylated amino group differs from that calculated by Holmquist & Schroeder (1966a) because they assumed that mobility depended upon charge alone and was independent of molecular weight. The mobilities, however, agree well with those that they reported for the peptide released from haemoglobin A_{1c}. The pK of 5.6 for the glucosylated amino group of Glc-Val-His is close to that of 5.85 inferred (with fewer assumptions) for Glc-Ile-Tyr (Table 1).

Chromatographic properties of Glc-Val-His

Fig. 1 shows that, as reported by Holmquist & Schroeder (1966a) for the peptide from haemoglobin A_{1c}, Glc-Val-His is eluted well before valylhistidine from a sulphonated polystyrene in a pyridine-acetate buffer. In view of the pK values estimated above, both peptides have almost identical charges at the pH of chromatography (5.0), so the difference in partition coefficient (about 5-fold, i.e. ΔG^0 for adsorption changed by 4.0 kJ/mol) is probably due to the changing of the partition of the molecule in favour of the aqueous phase (as opposed to the hydrophobic resin) by the sugar residue. This may be compared with the results of Barrett & Northcote (1965) who found, with a series of oligosaccharides that contained one residue

of galacturonic acid, that the addition of each neutral monosaccharide residue changed twofold the partition coefficient of the substance between the acetate of a basic resin and a buffer solution.

Formation and breakdown of Glc-Val-His in aqueous solution

No conversion of Glc-Val-His into valylhistidine or the reverse could be detected (by paper electrophoresis) after 2 days at 37°C in a solution of 1M-glucose-1M-pyridine-1M-acetic acid, but both processes occurred at 55°C. Lowering the concentration of acetic acid (or pyridine) much below 0.1M made equilibration very slow. A solution of 0.5M-glucose-1M-pyridine-0.1M-acetic acid (pH 6.2) was chosen for the quantitative determination at 50°C shown in Figs. 1 and 2. From this it appears that the dissociation constant of Glc-Val-His under these conditions is about 0.3M.

To check if there was anything peculiar about valylhistidine in this reaction, 5mg of isoleucyltyrosine was similarly incubated in 1ml of 0.4M-glucose-0.3M-pyridine-0.1M-acetic acid for 3 days at 55°C. Paper electrophoresis (Table 1) and chromatography on a sulphonic resin showed that a similar reaction had occurred; on chromatography in 0.15M-acetic acid-0.15M-pyridine buffer the presumed Glc-Ile-Tyr emerged at 1.7 column volumes and the unchanged isoleucyltyrosine at 7 column volumes. The Glc-Ile-Tyr amounted to 34% of the total of the two (judged by E_{280}). Under these conditions N^α-acetyl-lysine was glucosylated more slowly.

Fig. 1. *Chromatography of a mixture of valylhistidine and Glc-Val-His*

The sample was prepared by incubating valylhistidine (5 mg/ml) for 2 days at 50°C in 0.5 M-glucose–1 M-pyridine–0.1 M-acetic acid buffer, pH 6.2. A sample (0.5 ml) was mixed with 1 ml of 0.25 M-acetic acid and applied to a column (30 cm × 1.1 cm) of sulphonated polystyrene (Zeo-Karb 225 SRC 16) equilibrated and eluted with 0.3 M-pyridine–0.3 M-acetic acid at 60 ml · h⁻¹ · cm⁻².

Fig. 2. *Interconversion of valylhistidine and Glc-Val-His in aqueous solution*

Samples of valylhistidine (○), 5 mg/ml, or Glc-Val-His (●), 8 mg/ml, were incubated at 50°C in 0.5 M-glucose–1 M-pyridine–0.1 M-acetic acid buffer for various times. The fraction of the resultant mixture that was Glc-Val-His was determined by ion-exchange chromatography (Fig. 1).

Discussion

In all the properties reported, the substance prepared by treating valylhistidine with glucose in pyridine and acetic acid is identical with that described by Holmquist & Schroeder (1966a) as released from haemoglobin A_{1c}, but the hexose in the haemoglobin

has not been identified. These properties, and its optimum formation and hydrolysis at slightly acid pH, are consistent with its formulation as a glucosylamine (II) in equilibrium with an imine (I) (cf. Isbell & Frush, 1951, 1958; Ellis & Honeyman, 1955; Capon, 1969).

An alternative formulation which may be considered is stabilization of the imine by addition of either the nitrogen or oxygen atom of the first peptide bond to the imine carbon; a 4-imino-oxazolidine or a 4-imidazolidone structure would result. The former, however, is rapidly hydrolysed in cold water (Davis & Levy, 1951), while the latter, known in the compounds of acetone with oxytocin and vasopressin, is resistant to the strongly reducing conditions of sodium in liquid ammonia (Hruby *et al.*, 1968). Neither of these structures therefore seems likely in the present case.

The value of 0.3 M for the equilibrium constant for the dissociation of Glc-Val-His to valylhistidine and glucose in aqueous solution at pH 6.2 is only approximate because of the high concentrations of glucose and of buffer components. Raising the pH to 7.0 should decrease the dissociation constant by almost 9-fold (10-fold per unit) because the glucosylamine has a pK of 5.6 so that over the whole range of 6.2–7.0 it will be largely unprotonated and the free amine largely protonated. Hence a dissociation constant of well below 0.1 M is reasonably predicted at pH 7, even though failure of the reaction to occur at this pH makes direct determination impossible.

This result bears on the question of formation of haemoglobin A_{1c} (Holmquist & Schroeder, 1966b). A glycosyl group could be added to the amino group by reaction with either a free sugar or some more powerfully glycosylating agent (such as glucose 1-phosphate, UDP-glucose, etc.). Since haemoglobin A_{1c} forms about 6% of the total haemoglobin A (Clegg & Schroeder, 1959) and since the glucose concentration inside the human erythrocyte is about 5 mM (Somogyi, 1933) the dissociation constant would be 80 mM if glucose, haemoglobin A and haemoglobin A_{1c} were in equilibrium. The value found therefore means that no more powerful glycosylating agent than the blood glucose need be postulated. This method of biosynthesis could explain the indications that the concentration of haemoglobin A_{1c} is raised in diabetes (Rahbar, 1968; Trivelli *et al.*, 1970). A low degree of glucosylation may therefore possibly be expected also for other α -amino groups that are exposed to the blood sugar.

The equilibrium constant found is of the same order as that which may be estimated from the results of Haugaard *et al.* (1951) and Haugaard & Tumerman (1956) for the reaction of leucine or leucylglycine with glucose. They observed a comparatively rapid reaction at pH 7.7 and 24°C, in marked contrast with the present results. Since their method entailed using saturated solutions of the amino acid or peptide (0.17 M-leucylglycine) conditions were very different and general acid or base catalysis may have been significant.

Selection of the β - rather than the α -chains (Holmquist & Schroeder, 1966a; Bookchin & Gallop, 1968;

Efremov & Huisman, 1969) is not too surprising in view of the large difference between the environments of the ends of the two types of chain (Perutz, 1970). Since the reaction *in vitro* is too slow to measure at body temperature, catalysis, provided either by the local environment or by an enzyme, may be important in forming the glucosylamine *in vivo*. Conceivably the absence of glucosyl residues from the α -chains could be due to better rather than worse catalysis of the reaction in their case, so that the bond is hydrolysed as the protein is isolated.

The reaction of sugars with lysine residues is nutritionally important and has been studied in great detail (see Hodge, 1953; Ellis, 1963), although usually in somewhat dehydrating conditions. Under such conditions the α -amino groups of insulin react faster than its lysine residue (Schwartz & Lea, 1952), but lysine residues may sometimes react even in aqueous solution (Feeney *et al.*, 1964). In the present study *N*^α-acetyl-lysine reacted very slowly in aqueous solution, and it needed much higher temperatures than valylhistidine to react in pyridine and acetic acid. On the acid side of the pH optimum this is easily explained, since then the attack of the free amine on the sugar is likely to be rate determining (Capon, 1969). Since the α -amino groups will be less protonated, and indeed are among the most basic groups of proteins not overwhelmingly protonated at neutral pH (cf. Jencks, 1969, p. 69), they should react faster than ϵ -amino groups under these conditions. It is harder to predict the effect of basicity on the rate on the alkaline side, or indeed on the equilibrium constant of the reaction; the failure of ϵ -amino groups to react seems to be a kinetic rather than a thermodynamic effect.

This paper is dedicated to Academician A. E. Braunstein in honour of his 70th. birthday on 26 May 1972 with respect and affection.

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