THE CARBOHYDRATE MOIETY IN HEMOGLOBIN A_{1C} IS PRESENT IN THE RING FORM

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1. Introduction

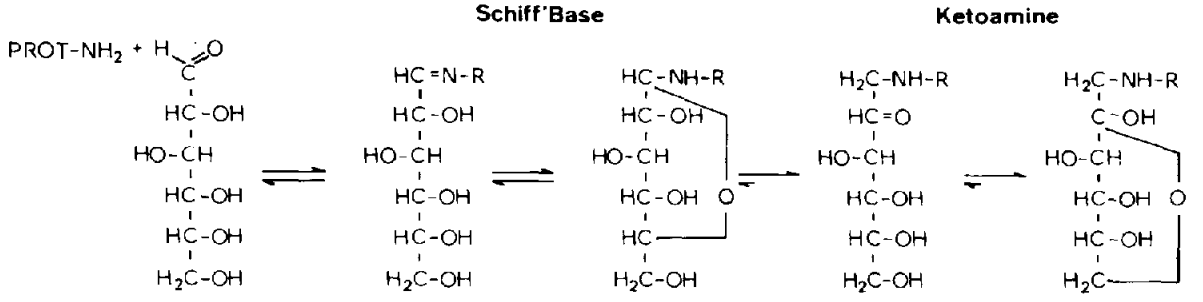
Non-enzymatically glycosylated proteins have gained wide interest as a contributing factor for the long-term secondary complications in subjects with diabetes mellitus. The most thoroughly investigated glycosylated protein is hemoglobin (HbA_{1C}) (reviews [1,2]). The measurement of the glycosylated hemoglobin content is a valuable criterion in delineating treatment of diabetic patients. Currently chromatographic (including HPLC) and chemical determination methods are used, both having slightly different targets [3–5].

The condensation between glucose and protein amino groups and the subsequent rearrangement reactions (scheme 1) take place at very different rates [6]. It is of prime importance to establish which of the glycosylated products of the reaction sequence is determined by a given method. To determine the configuration of the bound glucose moiety we have used phenylhydrazine as a reagent for keto-groups. The absence of detectable keto-groups in the 1-amino-1-deoxy-fructose moiety shows that these glyco-groups are in a ring structure.

2. Experimental

Purification of hemoglobin A and A_{1C} was done as in [7]. This preparation was used without further purification. Heme-free globin was obtained as in [8]. If the globin was still brownish, the precipitation step was repeated. Reaction of keto-groups with phenylhydrazine was done by a simplification of the procedure in [9]. Globin (10 mg, 0.6 mol) were dissolved in 1 ml 6 M guanidine in 10 mM phosphate (pH 7.0). To this solution 100 μl (~1 mmol) phenylhydrazine were added. After incubation for 30 min at 37°C the samples were passed through a Sephadex G-15 column (1.5 × 10 cm). Fractions containing the protein were brought to a concentration giving an absorption reading at 280 nm of ~1.1. Spectra between 260 and 550 nm were recorded for each sample.

The glyceraldehyde-modified hemoglobin A, which was used as control for keto-group detection, was obtained according to [9]. Globin from this preparation was difficult to dissolve in the guanidine–phosphate buffer. It was therefore freed from insoluble material by centrifugation prior to the incubation with phenylhydrazine.



Scheme 1

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The protein concentration was determined according to [10] using the protein assay kit by Bio-Rad. Globin served as standard protein.

3. Results and discussion

Chemical reactions with group-specific agents are in use to elucidate chemical [9] and stereochemical [11] structures of native and modified hemoglobins. The phenylhydrazine reaction was used in [9] to detect the keto-amine-group in glyceraldehyde-treated hemoglobin and to monitor the Amadori rearrangement of the glyceraldehyde-hemoglobin–Schiff'base adduct.

Here we applied the same reaction in order to search for keto-groups in the glycosylated hemoglobin fraction (HbA_{1C}). In fig.1 the UV–VIS spectra of the phenylhydrazine-treated globins A and at identical concentrations A_{1C} are shown. In both cases, no significant formation of hydrazone, which would absorb around 350 nm, was observed.

Increasing the temperature, the concentration of phenylhydrazine or the incubation time, did not lead to the formation of the hydrazone product. Globin from hemoglobin premodified with glyceraldehyde, however, exhibits after treatment with phenylhydrazine an elevated absorption around 350 nm, which is also found to a lesser extent in premodified globin A_{1C} after the same treatment (fig.2).

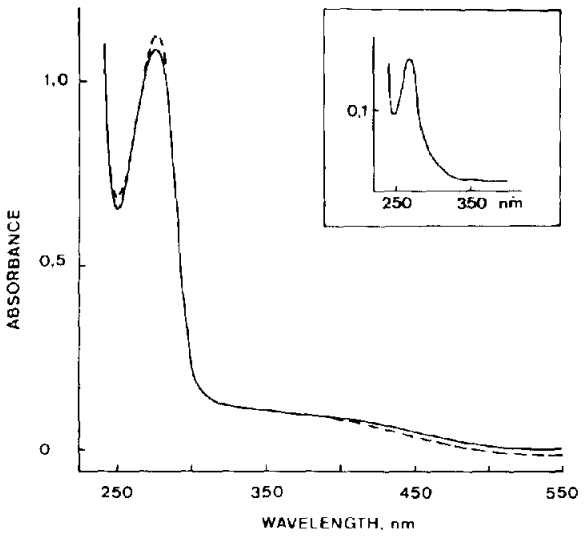


Fig.1. UV–VIS spectra of native globins after treatment with phenylhydrazine: (——) globin A; (– – –) globin A_{1C}. Inset: UV-Spectrum of a 0.001% phenylhydrazine solution in 0.1 M acetic acid.

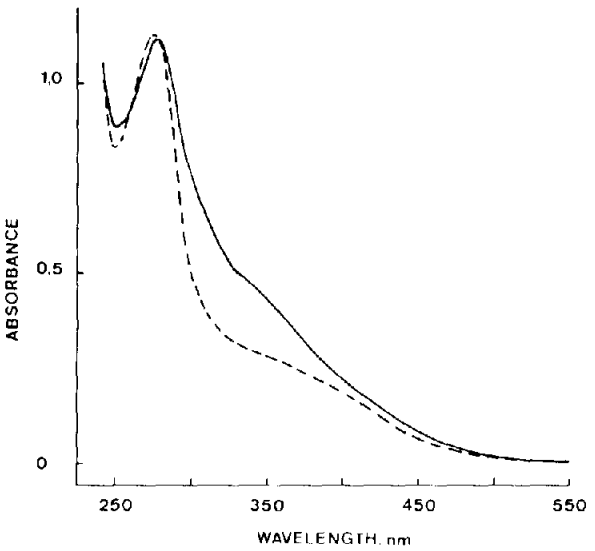
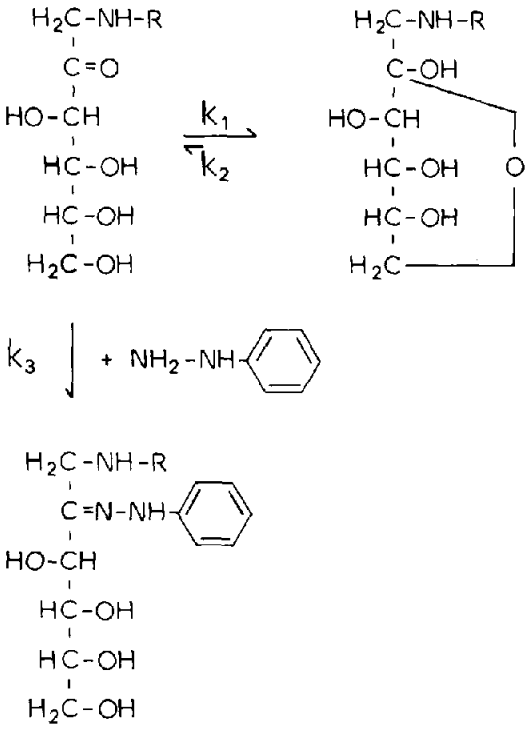


Fig.2. UV–VIS spectra of phenylhydrazine-treated globins, pre-reacted with glyceraldehyde: (——) globin A; (– – –) globin A_{1C}.

The samples were adjusted to the same protein concentration using extinction at 280 nm. Traces of unbound phenylhydrazine present in the sample would influence the absorption ratio 280/350 (fig.1 inset). Therefore the protein concentration was also determined colorimetrically. Since this determination gave the same protein concentration, contamination by phenylhydrazine can be excluded.

The finding of a difference in absorption at 350 nm between modified globin A and A_{1C} after phenylhydrazine treatment reflects the blocked α-amino-group at the N-terminus of the β-chain in hemoglobin A_{1C} which no longer binds glyceraldehyde. Consequently, ε-amino-groups of lysines as well as the N-terminal α-amino-group of the α-chain are the remaining candidates for these modifications [12].

Scheme 2 outlines the reaction sequence of the rearrangement from the open to the ring form of the carbohydrate moiety of HbA_{1C} (k_1 and k_2) and that of the reaction from phenylhydrazine with the keto-amine of the open form (k_3). The lack of detectable keto-groups in HbA_{1C} can be explained either by $k_1 \gg k_2$ or $k_3 \ll k_1$. The latter explanation seems unlikely since, even upon prolonged incubation of



phenylhydrazine with the protein, no hydrazone derivative could be demonstrated. Furthermore, in glyceraldehyde-treated hemoglobin, which does not have a rearrangement from an open to a ring form, keto-groups were easily detected with this method.

Other evidence, indicating the presence of a ring-formed glucose derives from [13]. In their work antibodies against native HbA_{1C} showed a much weaker binding to borohydride-reduced HbA_{1C} in which the glucose is present in an open form. Furthermore, in [14] upon periodate oxidation of the glycosylated hemoglobin, only 1 mol formaldehyde/mol glycogroup formed. Periodate treatment of an open glucose would liberate 2 molecules of formaldehyde.

These findings show that direct chemical demonstration of the protein-bound glucose is difficult. Chemical detection therefore requires drastic reaction conditions such as heating with acid or oxidation in order to detect the derivatives thereby formed.

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