NEW METHODS FOR THE PREPARATION OF HUMAN SEMI-HEMOGLOBIN

DISCUSSION ON FORMATION MECHANISM

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The preparation of a stable di-haem derivative of hemoglobin has recently been described by Banerjee and Cassoly (1967). The compound (semi-hemoglobin) has been studied extensively by Cassoly et al. (1967) who have shown it to possess the normal $\alpha_2\beta_2$ composition, the haems being situated exclusively on the α chains: the composition was described by the notation $(\alpha H)_2\beta_2$.*

The object of this note is to record new experimental facts which, while leading to new methods for the preparation of this hemoprotein, yield information on its genesis.

It may be recalled that semi-hemoglobin was originally isolated in small yield from a medium containing initially human methemoglobin and horse apomyoglobin which had reacted in the cold at pH 7 for a week. The reaction obviously involves haem transfer from methemoglobin to apomyoglobin, and should give rise to two new protein species, namely apohemoglobin and apomyoglobin. The reaction medium (at about 50% haem transfer) thus contained methemoglobin, metmyoglobin and their respective apoproteins in approximately

* Some evidences not to be discussed here now tend to show that semi-hemoglobin can exist also in a dissociated form $(\alpha H)\beta$.

equivalent concentrations. In view of the complexity of this reaction medium, it was not possible to propose any hypothesis on the mechanism of formation of semi-hemoglobin.

Some information bearing on this aspect has been gathered through the study of the following reactions.

I) Methemoglobin $(\alpha H)_2(\beta H)_2$ + apohemoglobin $\alpha\beta$

II) Isolated αchain (αH) + apohemoglobin $\alpha\beta$

The experiments have consisted in allowing these constituents to react and to look for the eventual appearance of semi-hemoglobin $(\alpha H)\beta$. The criteria for the identification of semi-hemoglobin are those described previously (1967), and may be resumed as follows.

The electrophoretic mobility and the spectral properties of semi-hemoglobin are characteristic; it can fix a quantity of haem equivalent to that already present; its splitting accomplished with PCMB, results in the precipitation of the haem-free chains; the latter are identified as the β chains by gel electrophoresis in 6 M urea and the coloured soluble fraction as the haem-containing α chains (αH).

I) The reaction between methemoglobin $(\alpha H)_2(\beta H)_2$ and its apoprotein $\alpha\beta$.

A solution containing these two proteins* (at 4 to 6 mg/ml each, phosphate buffer 0,1 M, pH 7, 0° C) was found to contain, already after 24 hours, a hemoprotein having the electrophoretic mobility of semi-hemoglobin. This constituent was isolated by fractional precipitation (ammonium sulfate at 62% saturation), followed by starch block electrophoresis.

Its analysis following the criteria stated above shows it to be none other than semi-hemoglobin which we had obtained earlier from a reaction mixture containing initially methemoglobin and apomyoglobin. This fact suggests that in this latter reaction the role of apomyoglobin was limited to the production

* It was not possible to show, even after 15 days, the formation of semi-hemoglobin when we used oxyhemoglobin or hemoglobin instead of methemoglobin.

of apohemoglobin from methemoglobin by haem transfer, and it is apohemoglobin which should participate in a reaction scheme for the formation of semi-hemoglobin.

Some further semiquantitative observations support this hypothesis. The amount of semi-hemoglobin that can be obtained from a given quantity of methemoglobin is about twice when it is allowed to react with apohemoglobin than when apomyoglobin is used (these two globins being both at the same concentration). This may be explained by the fact that when apomyoglobin is employed and when about 50% of haem is transfered, the concentration of the two reactive species, methemoglobin and apohemoglobin, are about half of those employed when the mixture is directly made with apohemoglobin. Moreover, the formation of semi-hemoglobin is much slower with apomyoglobin, being apparently limited by the slow rate of haem transfer.

II) Reaction between isolated α chains (αH) and apohemoglobin αβ.

This reaction* forms the basis of a new method for the preparation of semi-hemoglobin. Figure 1 shows the electrophoretic pattern of such a mixture (αH+αβ) when the material is applied immediatly after preparation (track a), and 48 hours later (track b). The track a shows two main bands corresponding to αH chains and apohemoglobin αβ. Ageing of the solution produces one main new band having the mobility of semi-hemoglobin. The constituent corresponding to this band is prepared as follows. The reaction mixture containing initially 10 mg/ml of apohemoglobin (αβ) and 5 mg/ml of αH chains in the oxy- or carboxy-form, in 0,1 M phosphate buffer pH 7, is left at 0° C for 48 hours. The fraction precipitating between 25 and 75% saturation by ammonium sulfate is then collected and purified by starch block electrophoresis. This hemoprotein is identified according to the criteria stated above as semi-hemoglobin. The advantage of this method of preparation lies in its yield which is higher than that described earlier.

* This particular part of this work has been developped starting from an experimental observation made together with Dr. E. Bucci.

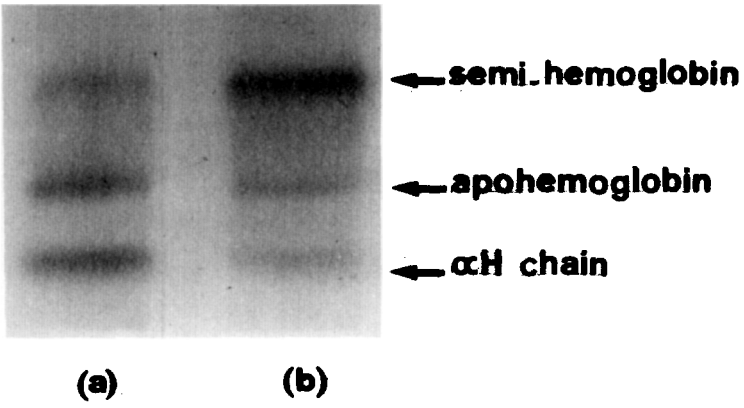

Figure 1. : Electrophoretic behaviour of a mixture of αH chains and apohemoglobin. Track a: a freshly prepared mixture. Track b: a mixture having evolved during 48 hours.

The formation mechanism of semi-hemoglobin by this method has been elucidated through experiments involving the utilization of marked αH chains carrying radioactive label in the protein moiety. These αH chains are prepared by the usual method of Bucci and Fronticelli (1965) from marked hemoglobin; the latter is obtained by in-vitro incubation, according to Lingrel and Borsook (1963), of red cells recovered from human blood containing 10% reticulocytes in presence of l-leucine and l-valine uniformly marked with C^{14}.

The labelled αH chains are reacted with unlabelled apohemoglobin for 48 hours in the cold and the reaction mixture in then analysed by starch gel electrophoresis. The bands corresponding to residual αH chains and the resulting semi-hemoglobin are quantitatively eluated in a given volume of water. The pure αH chains treated rigorously in the same manner constitutes the blank. The amount of radioactivity read on equal volumes of the test and blank solutions furnishes a basis for comparison. Figure 2 shows clearly that large amounts of radioactive label are transfered to semi-hemoglobin. Since the label is localized to the protein

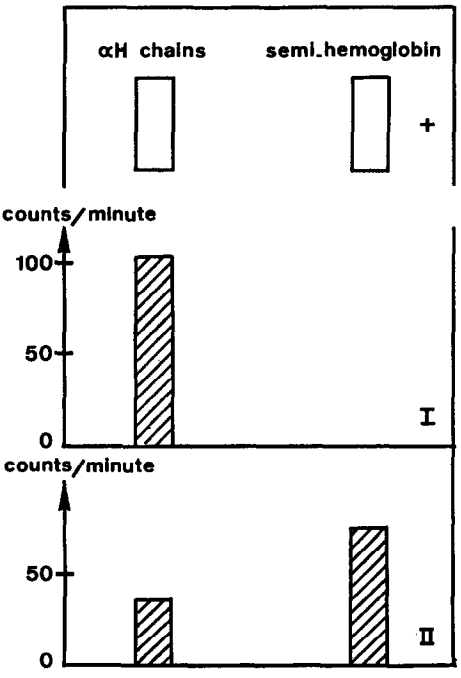

Figure 2. : Chain exchange between αH chains and apohemoglobin.
Top: schematic representation of the positions occupied by the αH chains and semi-hemoglobin in a concurrent run made on starch gel.

I) radioactivity measured (in counts/minute) on the eluate of αH band when radioactive αH chains only were used.

II) radioactivity measured on the eluates of the αH band and that of the semi-hemoglobin band when a 48 hours old mixture (αH + αβ) is analysed. The large transfer of radioactivity to semi-hemoglobin is quite evident.

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moiety of the αH chains, this can arise only if the free αH chains themselves entered into the composition of semi-hemoglobin. The reaction can then be formulated as follows:

(αH) chain + apohemoglobin ⟶ semi-hemoglobin + haem-free α chain

$$(\alpha^{*}H) + \alpha\beta \longrightarrow (\alpha^{*}H)\beta + \alpha$$

The scheme predicts the formation of haem-free α chains concurrently with that of semi-hemoglobin. This has in fact been demonstrated. The haem-free α chains were collected from the reaction mixture as a denatured precipitate at

25% saturation by ammonium sulfate and identified as such by electrophoresis in urea.

The results reported above show that a compound having the structure of semi-hemoglobin can be formed through diverse reaction paths;* it seems to originate whenever the apoprotein of hemoglobin is made to come in contact with the α chain, isolated or in the usual assembled form $(\alpha H)_2(\beta H)_2$.

The apparent stability of this structure implies a strong inter-chain association, between those who carry haem and those who do not. The formation mechanisms might possibly differ for individual reaction paths, but involves at least in one case the transfer of chains rather than the isolated haem groups.

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* A new semi-hemoglobin structure, the haem bearing chains being the β, has been obtained in the same way by chain transfer between the apohemoglobin and the isolated β chains; the properties of this hemoprotein have not yet been throughly studied.