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Immobilized human hemoglobin, a versatile matrix for analytical and biotechnological applications

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Abstract

The analytical and biotechnological applications of human hemoglobin immobilized covalently on CNBr–Sepharose 4B are reviewed. Hemoglobin is bound to the matrix as αβ dimers via either chain. The immobilized αβ dimers maintain the capacity to interact reversibly with soluble ones under conditions where the soluble protein is in self-association equilibrium. Under these conditions, therefore, immobilized dimers bind part of the soluble protein. In turn, the binding process can be used to assess the specific features of the equilibrium on solid-phase and to extract selectively hemoglobin from a variety of biological specimens of practical interest. A different application of immobilized αβ dimers concerns their use in the determination of the equilibrium and kinetic stability of the heme–globin linkage, a property that is directly correlated with the stability of the hemoglobin molecule. The advantages and limitations attendant the use of the immobilized protein relative to the soluble one are discussed.

Keywords: Hemoglobin

1. Introduction

The equilibria that take place in a human hemoglobin solution, namely the dissociation of the $\alpha_2\beta_2$ tetramer into subunits and the release of heme from the globin moiety, represent the starting point for the analytical and biotechnological applications of immobilized hemoglobin.

Dissociation of the hemoglobin molecule into subunits occurs in stages that lead to the formation of αβ dimers and of isolated α and β chains by means of rapidly attained equilibria. The first step in the dissociation gives rise to αβ dimers by cleavage of the so-called $\alpha_1\beta_2$ interface. A wealth of studies has shown that this process is affected by the presence of heme and non-heme ligands, pH and buffer composition [1]. The ligation and oxidation state of the heme are of special importance: the presence of ligands of ferrous heme and oxidation [2] favour dimer formation. The sensitivity to ferrous heme ligands is such that oxygen and carbon monoxide promote dissociation to significantly different extents, while deoxygenated hemoglobin is essentially not dissociated. Dimer formation is favoured also by an increase in ionic strength and by a departure from neutrality towards the alkaline or the acid region. Among non-heme ligands, the most remarkable effect is produced by organic and inorganic phosphates which bind in the crevice between the β chains and hence inhibit dimer formation [1]. The subsequent step of the dissociation process, in which αβ dimers give rise to isolated α and β chains, takes

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place only at extremes of pH [3], upon exposure to certain salt solutions [4], or after reaction of the masked SH groups (α_{104} and β_{112}) with *p*-chloromercuribenzoate [5] and therefore is of limited physiological relevance.

Dissociation of heme from the globin moiety does not occur under physiological conditions. However, the heme–globin linkage is considerably weakened upon oxidation of the heme iron. Hence, dissociation of heme from aquomet–hemoglobin occurs even in vivo as indicated by the appearance of the heme–albumin complex in patients with hemoglobinemia whenever the level of plasma hemoglobin exceeds the hemoglobin-binding capacity of plasma haptoglobin [6]. In aquomet–hemoglobin the rate of heme dissociation, which is usually taken as a measure of the heme–globin bond, is faster from β than from α chains and increases upon dissociation of the $\alpha_2\beta_2$ tetramer into αβ dimers [7–9]. At physiological temperatures, hemin loss is rendered effectively irreversible by the rapid precipitation of globin [6].

The main features of the subunit and heme dissociation equilibria just recalled bring out the key position occupied by αβ dimers. It follows that αβ dimers immobilized on a hydrophilic matrix provide an ideal and versatile material to both study these equilibria and exploit them for practical purposes.

1.1. Immobilized αβ dimers and the dimer–tetramer association equilibrium

Human hemoglobin is immobilized covalently on hydrophilic matrices like CNBr-activated Sepharose 4B as αβ dimers that are linked to the matrix via either chain [10]. The first property of immobilized αβ dimers which was recognized is that they maintain the capacity to interact in a specific and reversible way with soluble dimers under conditions where soluble hemoglobin is in self-association equilibrium. This observation contributed to establish the concept of subunit-exchange chromatography as an analytical and preparative tool [10,11]. As a result of the new equilibrium between soluble and immobilized αβ dimers, part of the protein that was initially in solution is retained on the solid-phase and the amount retained can be both calculated and measured easily. The calculation is based on the relevant equilibria in solution and on solid-phase:

$$2\alpha\beta \leftrightarrow \alpha_2\beta_2; \quad [\alpha\beta]^2 L_1 = [\alpha_2\beta_2]$$

$$\text{R-}\alpha\beta + \alpha\beta \leftrightarrow \text{R-}\alpha_2\beta_2; \quad [\text{R-}\alpha\beta][\alpha\beta] L_2 = [\text{R-}\alpha_2\beta_2]$$

where αβ and $\alpha_2\beta_2$ refer to the soluble protein, R-αβ is the immobilized dimer and R-$\alpha_2\beta_2$ the tetramer formed upon binding of a soluble dimer to an immobilized one, L_1 and L_2 are the association equilibrium constants for the formation of soluble and immobilized tetramers, respectively. In turn, the experimental determination may be carried out in frontal chromatographic experiments involving a column of immobilized αβ dimers, since the interaction on solid-phase leads to retardation in the elution of hemoglobin with respect to an inert protein [11]. The retardation depends on the relative concentrations of the soluble and immobilized protein and on the tendency of soluble αβ dimers to associate with themselves and with those on solid-phase. Comparison of the calculated and measured amounts of retained protein therefore provides valuable insight into the association capacity of the immobilized αβ dimers; in addition, it allows determination of the so-called effective concentration of immobilized hemoglobin, i.e., of the concentration available for interaction with the soluble protein [12,13]. Under all coupling conditions tested, which include immobilization at different pH values in the range 6–9 to achieve a certain selectivity in the coupling step, and presence or absence of oxygen to assess the influence of the initial state of association, the effective concentration of immobilized dimers is about one half the analytical one and their associating capacity is impaired two- to three-fold relative to the soluble protein. However, when coupling occurs in the deoxygenated state, the population of immobilized dimers is more homogeneous with respect to their associating capacity [12]. Interestingly, the immobilized αβ dimers maintain their sensitivity to chloride ions, but loose that to phosphate ions, consistent with the different nature of the phosphate and dissociation-linked chloride binding sites [14] and with coupling of hemoglobin to the matrix via amino groups near the organic phosphates binding region.

In the preparative fashion, advantage is taken from the fact that retardation in the elution of hemoglobin from a column of immobilized αβ dimers is largest

when the concentration in solution is low [11]. Such columns have been used as efficient affinity matrices to separate hemoglobin from myoglobin for the purpose of determining myoglobin in cardiac and skeletal muscle [15], to remove cell-free hemoglobin from biological specimens [16], and to purify hemoglobin, cross-linked between the α or the β chains to be used as an oxygen carrier, from any dissociable hemoglobin that passes rapidly through the kidneys and hence has adverse effects [17]. These preparative applications furnished also interesting analytical information. Thus, the work on biological specimens showed that immobilized hemoglobin does not recognize the hemoglobin–haptoglobin complex, formed by interaction of haptoglobin with αβ dimers; a clear demonstration that the same $\alpha_1\beta_2$ subunit interface that is cleaved upon dimer formation is involved in the interaction with haptoglobin. Conversely, the experiments with cross-linked hemoglobin furnished unexpected data on the cross-linking reaction itself as they demonstrated for the first time formation of cross-links within αβ dimers. In addition they showed that the potentiality of hemoglobin affinity columns is enhanced by the interaction between dimers from different species which gives rise to hybrid hemoglobin molecules; thus, for example, a column of human hemoglobin can be used to purify cross-linked bovine hemoglobin.

1.2. Immobilized αβ dimers and heme dissociation equilibrium

Due to the high stability of the holoprotein, the heme–globin equilibrium is usually assessed by monitoring the heme transfer reaction to an acceptor protein, a study which is hampered by the formation of free, precipitable globin [6]. Immobilized αβ dimers used as heme donors appeared advantageous in this respect as they are stable after heme depletion. In principle, they offered other advantages as they do not undergo association–dissociation reactions thus facilitating data analysis and, in addition, can be separated easily from the acceptor protein, thus allowing an easy spectrophotometric determination of the heme-transfer reaction under any experimental condition.

The observation that immobilized αβ human hemoglobin dimers, just like the soluble protein, transfer heme to human albumin only when the heme iron is oxidized, indicated that immobilization does not alter the heme environment significantly. This contention was confirmed in a detailed study of the heme transfer reaction to human albumin [8]. The heme transfer reaction can be schematized as follows:

$$R\text{-}\alpha H\beta H + A \leftrightarrow R\text{-}\alpha H\beta + AH$$

$$R\text{-}\alpha H\beta + A \leftrightarrow R\text{-}\alpha\beta + AH$$

where R-αHβH, R-αHβ and R-αβ are the immobilized αβ dimers, H stands for heme, and A for albumin. Heme transfer to albumin is not complete, since the affinity for heme of human albumin and αβ dimers is comparable [18,8]. The ease with which the heme–albumin complex can be separated from the matrix allows one to follow the kinetics of the heme transfer reaction and in addition to estimate the partition constant of the heme between donor and acceptor. As for hemoglobin in solution the kinetics of heme transfer displays two distinctly separated phases; the faster one reflects heme release from the β chains, the slower one release from the α chains. Although the absolute rates of heme loss from α and β chain in immobilized αβ dimers are slightly faster than in solution, the ratio between the heme loss rates is similar [8,9]. In solution, association of αβ dimers into $\alpha_2\beta_2$ tetramers results in the decrease in the rate of heme loss from β chains indicating that formation of the so-called $\alpha_1\beta_2$ interface increases the strength of the heme–globin linkage in this chain type. In contrast, formation of the so-called $\alpha_1\beta_1$ interface affects the heme–globin linkage in the α chains. This is clearly shown by the observation that isolated α and β hemoglobin chains transfer heme to albumin at a similar rate. Interestingly, the isolated hemoglobin chains transfer heme not only when oxidized, but also when in the reduced CO-bound form, an indication that a major rearrangement takes place in the heme pocket of the reduced protein upon assembly of unlike chains such that in practice heme dissociation cannot be measured.

The natural extension of these studies is the use of immobilized αβ dimers to determine variations in the stability of the heme–globin linkage attendant mutations or chemical modifications of the hemoglobin molecule. In this connection, two drawbacks can

be envisaged: one is due to the small increase in the rates of heme release relative to soluble dimers that is indicative of small alterations produced by the immobilization reaction, the other lies in possible limitations in the availability of mutated or modified hemoglobins. To this end preparation of immobilized heme acceptors is under study.

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