Immobilized apo-myoglobin, a new stable reagent for measuring rates of heme dissociation from hemoglobin

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Abstract Apo-myoglobin covalently linked on CNBr-activated Sepharose 4B is proposed as a new heme acceptor for investigating the heme transfer reaction from hemoproteins. Immobilized apo-myoglobin has the desirable properties of an ideal heme acceptor in that it is characterized by a high affinity for ferric heme, a high stability towards denaturation even at physiological temperatures and can be lyophilized for long-term storage. The study of heme release from myoglobin at pH 5.0 and 37°C indicates that heme affinity is increased at least 10-fold relative to the soluble protein. Experiments with human hemoglobin allowed the estimation of the heme release rates from both α and β chains and brought out the greater temperature sensitivity of the α chain heme-globin linkage.

Key words: Heme transfer; Immobilized apomyoglobin; Ferric hemoglobin

1. Introduction

The affinity of heme for the globin moiety is an important parameter in the description of hemoproteins [1,2]. It is relevant also in the design of hemoglobin mutants or chemically modified hemoglobins for biotechnological applications due to the instability of the globin moiety and to the adverse effects of free heme on biological membranes. In hemoglobins and myoglobins the high stability of the holoprotein under physiological conditions permits only the indirect determination of the heme-globin linkage by means of experiments in which heme is transferred to an acceptor protein. In human hemoglobin (HbA) the transfer reaction can be measured only in the oxidized, aquomet derivative due to the significant weakening of the proximal histidine-iron bond induced by heme oxidation [1]. The kinetics of heme transfer is dominated by the rate of heme dissociation from the donor protein provided the heme acceptor is endowed with a high affinity for heme [2–4]. An ideal heme acceptor, in addition to having this property, should be amenable to long-term storage and should be resistant to denaturation for several days at room or better at physiological temperatures since heme dissociation reactions are relatively slow.

Mutants of sperm whale myoglobin which retain the high affinity for heme characteristic of the native protein have been proposed recently as reagents for measuring heme dissociation from myoglobins and hemoglobins. However, a practical limit to their use is set by the intrinsic instability of globins at room temperature, even though the tendency to precipitate can be partially overcome by the addition of sucrose to the solution [2].

In this paper we show that apomyoglobin immobilized covalently on Sepharose 4B has all the properties of an ideal heme acceptor as it is stable at 37°C for several days, can be freeze-dried for long-term storage and permits the quantitative analysis of the heme dissociation reaction from hemoglobin and myoglobin.

2. Materials and methods

2.1. Preparation of immobilized myoglobin

Horse skeletal muscle myoglobin (Mb) was purchased from Sigma Chemical Co. (USA) and was immobilized on CNBr-activated Sepharose 4B (Pharmacia Biotech Inc., Uppsala, Sweden) in the oxidized state. The coupling reaction was carried out in 0.1 M sodium bicarbonate, pH 8.3. The suspension was stirred for 1 h at room temperature and washed with coupling buffer as described in [4]. The concentration of immobilized protein was typically around 12 mg per ml of packed resin; it was determined on every preparation using a 2 mm flow cell and protein-free gel in the reference cell to minimize the effect of turbidity [5].

Myoglobin concentration was determined spectrophotometrically at 555 nm on the deoxygenated protein, using the molar absorptivity of 11 800 M^{-1} cm^{-1} [6].

2.2. Preparation of apo-Mb from immobilized Mb

Immobilized apo-Mb was prepared using the methyl-ethyl-ketone method of Yonetani [7]. Immobilized myoglobin, usually 0.8 ml, was equilibrated with water at 4°C, placed in a test tube and brought to acid pH by addition of 0.1 M HCl. Cold methyl-ethyl-ketone (about 10 ml) plus 0.3 ml of 1.0 M HCl was immediately added to the test tube. After vigorous shaking, the solution was left to stand in the cold for a few minutes. The process was repeated twice to achieve a good extraction of heme. Thereafter, the resin was washed extensively with water on a filter funnel and lyophilized in the presence of 10% sucrose.

The concentration of immobilized apo-Mb was determined spectrophotometrically with the same procedure described above for the holoprotein on the basis of a molar absorbance at 280 nm of 15 900 [8].

2.3. Heme release experiments

A constant amount of lyophilized immobilized apo-Mb (200 mg corresponding to 0.8 ml of packed gel) was equilibrated with the desired buffer containing 0.3 M sucrose, mixed in a test tube with 2.5 ml of buffer containing methemoglobin or metmyoglobin at a known concentration and placed in a water bath thermostatted at 20 or 37°C. At established times, a filter sampler (Porex Medical, USA) was pressed into the test tube to separate the solid and the liquid phase, whose absorbance was measured in a Cary 219 spectrophotometer between 650 and 350 nm.

The decrease in the absorbance of hemoglobin in solution due to the transfer of heme to immobilized apo-Mb can thus be followed as a function of time, without interference from the immobilized protein [4]. The buffer used in the heme release experiments was 0.15 M sodium acetate at pH 5.0. The buffers contained 0.3 M sucrose in order to stabilize the soluble apoprotein formed during the heme transfer reaction [2].

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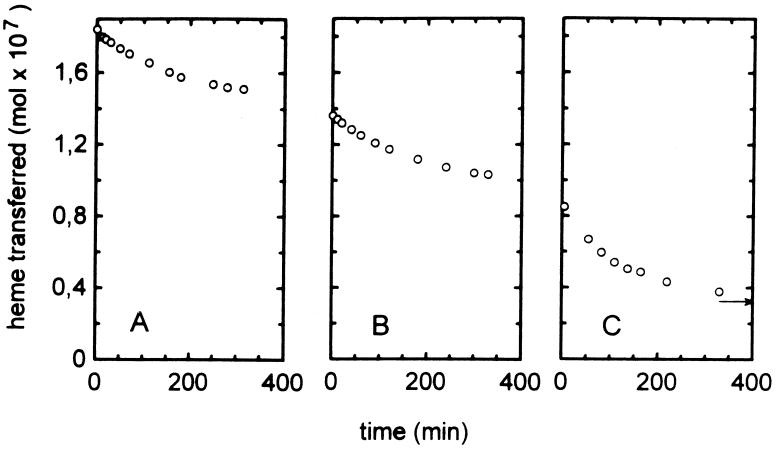

Fig. 1. Heme transferred from soluble to immobilized apo-Mb at pH 5.0 and 37°C. The kinetics of heme transfer was followed after three successive additions (A, 0.36×10^{-7} mol; B, 0.36×10^{-7} mol; C, 7.2×10^{-7} mol) of soluble ferric myoglobin to immobilized apo-Mb (1.76×10^{-7} mol). The amount of heme bound to the immobilized protein is reported in the abscissa. The arrow indicates the final equilibrium point, 3 days after the last soluble myoglobin addition.

Heme transfer from hemoglobin to immobilized apo-Mb was analyzed according to the following reaction scheme on the assumption [2,4] that the concentration of free heme in solution is constant ($dH/dt = 0$).

$$\alpha H\beta H + Mb \xrightarrow{k_{\beta d}} \alpha H\beta + MbH \quad (1)$$

$$\alpha H\beta H + Mb \xrightarrow{k_{\alpha d}} \alpha\beta H + MbH \quad (2)$$

$$\alpha_2 H\beta_2 H + Mb \xrightarrow{k_{\beta t}} \alpha_2 H\beta_2 + MbH \quad (3)$$

$$a_2 H\beta_2 H + Mb \xrightarrow{k_{\alpha t}} \alpha_2\beta_2 H + MbH \quad (4)$$

$$\alpha_2\beta_2 \overset{K_{2,4}}{\Leftrightarrow} 2\alpha\beta \quad (5)$$

where $\alpha\beta$ and $\alpha_2\beta_2$ are the hemoglobin dimers and tetramers respectively, H stands for heme and Mb for myoglobin; $k_{\alpha t}$ and $k_{\beta t}$, $k_{\alpha d}$ and $k_{\beta d}$ are the kinetic dissociation constants of heme from α and β chains in tetramers and dimers and $K_{2,4}$ is the association equilibrium constant for the dimer-tetramer equilibrium. The following approximations and assumptions have been made in order to fit the experimental data to the scheme: (1) the rate of heme release from myoglobin has been assumed to be equal to zero in view of its very low value; (2) the rates of heme release from α chains in dimers and tetramers have been taken to be equal [2,9]; (3) the equilibrium constant $K_{2,4}$ has been fixed to the experimental value determined independently; (4) the rates at which dimers and tetramers reequilibrate are fast relative to the heme release ones [2,9]. Within this framework, only three kinetic constants, $k_{\beta d}$, $k_{\beta t}$ and k_{α}, were allowed to float during the fitting procedure. The data fitting was carried out on the whole set of curves obtained under the same experimental conditions at different protein concentrations using the Matlab Program (The Math Works Inc., Natick, MA) on a PC IBM 486.

2.4. Dimer-tetramer equilibrium of ferric HbA

The equilibrium constant for the dimer-tetramer association reaction was determined by means of gel filtration experiments on a Sephadex G75 column in 0.15 M acetate at pH 5.0 plus 1 mM EDTA, at 10°C [10]. The flow rate was 12 ml/h. The elution profile was monitored by following the absorbance value at 540 or 405 nm with a Jasco 7800 spectrophotometer equipped with a thermostatted flow cell. The column was calibrated using horse heart myoglobin for the elution volume of the monomer, V_1, and hemoglobin cross-linked between the β chains with 3,5-dibromosalicylfumarate for the elution volume of the tetramer, V_4 [11]. The elution volume of the dimer, V_2, was calculated assuming a linear relationship between elution volume and the logarithm of molecular weight.

3. Results

3.1. Stability and heme binding capacity of immobilized apo-Mb

The heme binding capacity of immobilized apo-Mb was tested at 37°C in acetate buffer 0.15 M at pH 5.0. A sample (0.8 ml at 2.2×10^{-4} M) of immobilized apo-Mb was titrated with three aliquots of soluble ferric myoglobin containing 4/5 of the total heme binding capacity of the matrix. The kinetics of heme transfer from soluble to immobilized Mb was followed spectrophotometrically over a period of 9 days (3 days for each aliquot). The Mb stock solution was kept at 4°C throughout the experiment. All the heme added is transferred to the immobilized protein (Fig. 1). The observed rate constant for heme release from soluble myoglobin was $3.5\pm1.1\times10^{-4}$ s^{-1} for the first two aliquots, in very good agreement with the value obtained by Hargrove et al. [2] using soluble myoglobin mutants as heme acceptor, namely $2.8\pm1.4\times10^{-4}$ s^{-1}. The rate of heme release from the third aliquot, i.e. from soluble Mb kept at pH 5.0 and 4°C for 6 days, was significantly higher ($8.8\pm2.4\times10^{-4}$ s^{-1}), an indication of the ageing of the soluble protein which was not apparent in the optical spectrum.

As a further proof that the immobilization reaction does not produce significant alterations in the heme environment, the spectrum of immobilized Mb formed at the end of the heme transfer reaction was measured. It corresponds to that of the soluble protein and in addition displays the same pH dependence (data not shown).

3.2. Rates of heme release from met-HbA

The rate of heme release from met-HbA was measured at pH 5.0, at 20 and 37°C. In these experiments the reaction was followed at a number of hemoglobin concentrations ranging between 3.7×10^{-6} M and 7.5×10^{-5} M (heme) using a fixed amount of immobilized apo-Mb (0.8 ml at 2.2×10^{-4} M). The time course of the heme release reaction is shown in Fig. 2. In all kinetic records a clearly biphasic behavior is observed, the fast phase represents heme release from the β chains and the slow phase heme release from the α chains [1,2,4,9]. After 3 h, virtually all the heme is transferred to immobilized apo-Mb at 37°C, whereas at 20°C between 20 and 40% of the heme

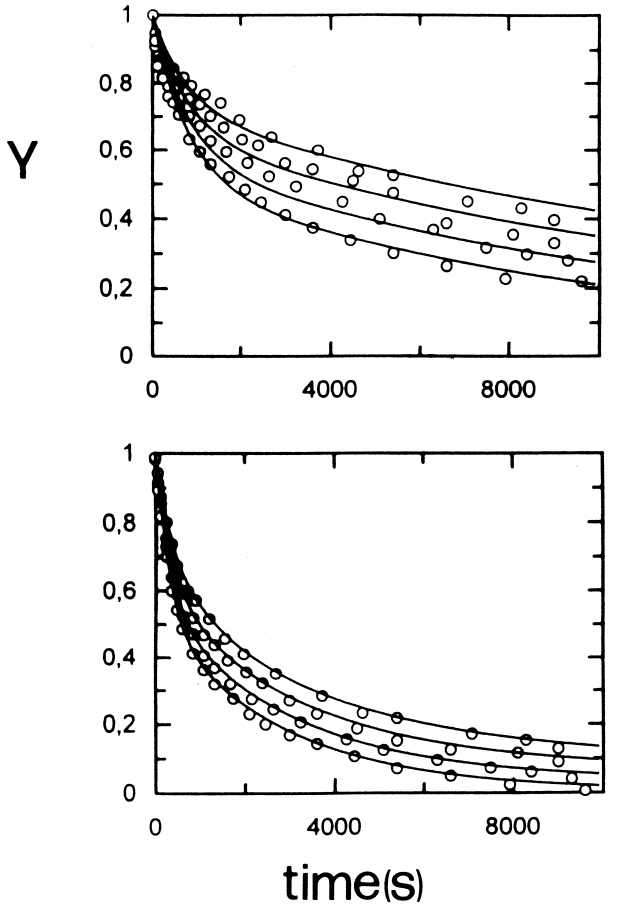

Fig. 2. Kinetics of heme transfer from ferric HbA to immobilized apo-Mb at pH 5.0. Y represents the fraction of heme remaining on the hemoglobin molecule. Temperature: top, 20°C; bottom, 37°C. Hemoglobin concentration (molar per heme), from top to bottom: top, 7.5×10^{-5}, 3.4×10^{-5}, 1.2×10^{-5}, 0.5×10^{-5}; bottom, 6.6×10^{-5}, 3.3×10^{-5}, 1.65×10^{-5}, 0.9×10^{-5}. Continuous lines represent the fitted curves according to the scheme described in Section 2. The dimer-tetramer association constants was taken as 1×10^{4} M^{-1} at pH 5.0 on the basis of independent measurements.

remains bound to soluble hemoglobin depending on its concentration.

The analysis of the heme transfer data requires knowledge of $K_{2,4}$, the dimer-tetramer equilibrium association constant, since it is well established that heme is released from hemoglobin dimers at a faster rate than from tetramers [4,9,12]. In turn, this feature is reflected in the concentration dependence of the heme release kinetics as apparent in Fig. 2. The dimer-tetramer association constant obtained by gel filtration is $1.0\pm0.2\times10^{4}$ M^{-1} at pH 5.0 and 10°C. No significant changes in the values of $K_{2,4}$ have been reported in the range of temperatures used and no correction for the $K_{2,4}$ values was applied [13,14]. The value obtained for $K_{2,4}$ was used as a fixed parameter for the data fit of Fig. 2, as described in Section 2. The calculated rates of heme release from the individual chains in dimers and tetramers bring out that the temperature dependence is significantly stronger for α than for β chains (Table 1).

Table 1
Rates of heme release from met-HbA to immobilized apo-Mb at pH 5.0

	20°C	37°C
$k_{\beta d}$	18 ±8	72 ±8
$k_{\beta t}$	3.2 ±1.0	10 ±2
k_{α}	0.98±0.15	7.3±3

The values of the rate constants were obtained with the fitting procedure described in Section 2. All rate constants are given in $s^{-1}\times10^{4}$.

4. Discussion

The present results indicate that apo-Mb linked covalently to CNBr-activated Sepharose displays all the properties of an ideal heme acceptor to be employed for the determination of the heme-globin affinity in myoglobins and hemoglobins. Immobilized apo-Mb not only displays an unaltered heme binding capacity, but is characterized by a much higher thermal stability relative to the soluble protein. The latter feature is a general consequence of covalent immobilization and hence is not unexpected. Quite surprisingly, however, the increased overall stability of immobilized apo-Mb also results in a very significant increase of the affinity for heme. This can be estimated to be at least one order of magnitude relative to the soluble protein, since at pH 5.0 and 37°C immobilized apo-Mb is capable of extracting heme from soluble Mb even when the molar excess is very slight (Fig. 1). The data in Fig. 1 also show that the apo-Mb matrix enables one to appreciate small, spectrally silent alterations in the heme-globin linkage such as those deriving from ageing of a Mb solution stored for a week at 4°C and pH 5.0. This characteristic renders the apo-Mb matrix an ideal acceptor for the study of the effects of mutations. In addition, the apo-Mb matrix can be easily lyophilized, stored at 4°C and employed when desired.

Immobilized apo-Mb was also used to study heme release from oxidized HbA with the aim of setting up a convenient assay that would allow analysis of complete time courses of the reaction. At pH 5.0, as expected on the basis of the data in Fig. 1 just discussed, all the heme is transferred from HbA to the immobilized acceptor after 3 h at 37°C and after slightly longer times at 20°C. The rate constants for heme release reported in Table 1 confirm the well established dependence of heme release from β chains on the position of the dimer-tetramer equilibrium [4,9,12] and in addition clearly show that the heme-globin linkage in α and β chains displays a significantly different temperature sensitivity. Thus, an increase in temperature between 20 and 37°C leads to a greater increase in the rate of heme release from α than from β chains. It is of interest to compare the data in Table 1 with those obtained under the same experimental conditions on micromolar HbA concentrations by Hargrove et al. [2] using a mutant Mb as heme acceptor. The values for the β chains, which pertain to dimers given the value of $K_{2,4}$, are in remarkably good agreement (16×10^{-4} and $55\pm13\times10^{-4}$ s^{-1}, respectively, at 20 and 37°C), whereas the α chains values are significantly lower (0.15×10^{-4} and $0.8\pm0.5\times10^{-4}$ s^{-1}, respectively, at 20 and 37°C), especially at the higher temperature. The reason for the discrepancy is likely to lie in the difficulties arising from instability of globins at physiological temperatures which easily leads to artifacts even in the presence of sucrose as a stabilizing agent. The use of the apo-Mb matrix abolishes denaturation of one of the two partners in the reaction, the heme acceptor, whereas in the Hargrove et al. system both partners are subject to denaturation. In this connection it is worth recalling that αβ dimers bound to Sepharose 4B have proven

to be extremely useful heme donors in the study of the heme-globin affinity in HbA. In fact, their use brought out for the first time that the heme-globin linkage in the α chains is stabilized by interactions between unlike chains at the $\alpha_1\beta_1$ interface, whereas heme binding to the β chains depends on interactions at the $\alpha_1\beta_2$ interface [4]. However, comparison with data in solution [4,9] shows that the immobilization reaction leads to a roughly three-fold increase in the rates of heme release, a difference that does not warrant the use of the αβ matrix to assess differences produced by site specific mutations or chemical modifications. The apo-Mb matrix, a stable, high affinity, lyophilizable heme acceptor, that is sensitive to small changes in the heme environment, lends itself naturally to this type of measurement.

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