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Biophysical Chemistry 51 (1994) 53–57

Biophysical Chemistry

The dimer–tetramer equilibrium of recombinant hemoglobins. Stabilization of the $\alpha_1\beta_2$ interface by the mutation β(Cys112 → Gly) at the $\alpha_1\beta_1$ interface

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(Received 18 October 1993; accepted 14 January 1994)

Abstract

The dimer–tetramer association constants of several recombinant human hemoglobins (in the CO form) have been measured by differential gel filtration. Recombinant human hemoglobin prepared from recombinant β-chains, and mutant hemoglobins where the substitution was on the surface, β(Thr4 → Asp), in the heme pocket, β(Val67 → Thr), at the 2,3-DPG binding site, β(Val1 → Met + His2del), had a twofold smaller association with respect to natural hemoglobin. In a mutant at the $\alpha_1\beta_2$ interface, β(Cys93 → Ala), the association constant was decreased three-fold. Conversely, in a mutant at the $\alpha_1\beta_1$ interface, β(Cys112 → Gly), the association constant was two- and four-fold increased with respect to natural and recombinant human hemoglobin. These differences are energetically very small, consistent with the correct folding of the recombinant hemoglobins. The stabilization of the tetrameric structure by a mutation at the $\alpha_1\beta_1$ interface indicates that structural changes at this interface can be propagated through the protein to the $\alpha_1\beta_2$ interface and, thereby, exert an effect on the allosteric equilibrium.

Key words: Recombinant hemoglobin; Subunit assembly; Differential gel filtration

1. Introduction

In this paper we report a study of the dimer–tetramer association of recombinant hemoglobins. These proteins have been obtained using mutagenesis and a gene expression system which allows the construction of wild-type human hemoglobin (HbA-Wt) with the identical amino acid sequence of natural human hemoglobin (HbA) [1], devoid of the initiating methionine residue in the α- and β-chains, as is the case for other expression systems [2,3]. In oligomeric systems, the association of protein subunits entails the establishment of precise interactions between the subunits, and hence is highly dependent upon the structural

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Abbreviations: HbA natural human hemoglobin; HbA-Wt human hemoglobin from recombinant β-chains.

SSDI 0301-4622(94)00028-I

integrity. Therefore, the determination of parameters associated with the assembly process of recombinant proteins provides a sensitive method to assess the correctness of tertiary folding, and also can be used to probe the effect of amino acid substitutions on the tertiary and quaternary structure.

Tetrameric hemoglobin has two structurally distinct interfaces between unlike subunits. These are designated $\alpha_1\beta_1$ and $\alpha_1\beta_2$. The $\alpha_1\beta_2$ interface is stabilized by fewer intersubunit contacts than the $\alpha_1\beta_1$ interface; the dissociation of the tetramer into $\alpha\beta$ dimer occurs with cleavage of the $\alpha_1\beta_2$ interface. The equilibrium between dimers and tetramer is rapidly reversible and is ligand linked. The $\alpha_1\beta_2$ interface makes a different set of intersubunit contacts in oxy compared to deoxy hemoglobin, and can be considered to be a principal regulatory site in the cooperativity in hemoglobin [4]. The $\alpha_1\beta_1$ interface retains the same contacts in the two quaternary structures [5].

The dimer–tetramer association constants of recombinant hemoglobins and of natural HbA have been compared directly by means of differential gel filtration. This technique enables the detection of 1–2% differences in elution volume and hence is ideally suited to precisely measure small differences in the quaternary assembly of related multimeric proteins [6,7]. In previous studies on recombinant hemoglobins the dimer–tetramer association had been estimated indirectly from oxygen binding isotherms [8].

All the recombinant hemoglobins investigated had correct tertiary and quaternary folding, as evidenced by the small differences in the dimer–tetramer association with respect to natural HbA. Mutants with amino acid substitution on the surface of the molecule, β(Thr4 → Asp), in the heme pocket, β(Val67 → Thr), or at the 2,3-DPG binding site, β(Val1 → Met + His2 → deleted), had the same association constant as the HbA-Wt; two-fold smaller than natural HbA. A mutant at the $\alpha_1\beta_2$ interface, β(Cys93 → Ala), was slightly more dissociated; however, a mutant at the $\alpha_1\beta_1$ interface, β(Cys112 → Gly), was almost four fold more associated than HbA-Wt. This latter result indicates structural communication between the two interfaces suggesting that both interfaces are involved in the regulation of the allosteric properties of hemoglobin.

2. Materials and methods

2.1. Plasmid construction and expression

A *Hin*dIII fragment of the human β-globin gene from pJK05 [1] was cloned into the *Hin*dIII site of M13MP18 for mutagenesis. The method of Kunkel et al. [9] from the BioRad mutagenesis kit was utilized to alter the human β-globin gene. The following oligonucleotides were used for the corresponding mutations: (1) β(Cys93 → Ala) 5′-TGC AGC TTG TCA GCA TGC AGC TCA CTC-3′; (2) β(Cys112 → Gly) 5′-CAG CAC ACC GAC CAG CAC-3′; (3) β(Val67 → Thr) 5′-GGC AAG AAA AGC CTC CGG GCC-3′ [10] (4) β(Thr4 → Asp) 5′-AAG CTG CAC CTC GAT CCT GAG-3′. This latter oligonucleotide was designed to generate the mutation β(Val98 → Leu). Of the 21 bases, 19 are complementary to the intended region and 16 to the region at the 5′-end. DNA from five placques was isolated and sequenced; all were identical having the sequence CTC GAT, instead of CTG ACT, at the third and fourth codons. The remainder of the globin sequence was identical to that of the native cDNA. β(Val1 → Met + His2 → del) was generated by the PCR method using pJK05 linearized with the *Bgl*II as the template. The mutagenic primer, 5′-CTT AAA ATG AAC CAT GGC GAT CGA AGG TCG TAT GCT GAC TCC GGA GGA GGA-3′, changed the Glu and Arg codons of the factor *X* recognition sequence from GAG to GAA and AGG to CGT respectively, as well as replacement of the Val1 codon, GTG, with ATG and elimination of the CAC His2 codon. The other primer for PCR was complementary to the 3′-end of the gene and contained an *Eco*RV recognition sequence. The PCR product was gel purified, cleaved by *Nco*I and *Eco*RV, and gel purified again. Mutated human β-globin gene was transferred back into the parent vector (pJK05) using the restriction enzymes *Nco*I and *Eco*RV. *E. coli* strain AR120 was transformed

with each of these plasmids. Induction of the protein, reconstitution into hemoglobin and purification, was done as previously described [1].

2.2. Gel filtration

These experiments were performed as described by Chiancone et al. [11]. A column (45 × 1.0 cm) of Sephadex G 75 (Pharmacia, Uppsala, Sweden) was employed at a constant flow rate of 12 ml/h and a temperature of 8.5°C. All experiments were carried out on the carbonmonoxy hemoglobin derivatives in a pH 7.0 buffer containing 0.01 M sodium tetraborate, 0.01 M NaH_2PO_4, 0.1 M NaCl and 1 mM EDTA. The buffer was saturated with CO, resulting in about 10^{-3} M CO. The hemoglobin solutions were prepared by adding a maximum of 0.5 ml of oxy-hemoglobin stock solution to 40 ml of CO saturated buffer. The buffer and hemoglobin resevoirs were layered with about 4 cm of paraffin oil. The elution profiles were monitored in the Soret region with a Jasco 7800 spectrophotometer equipped with a thermostatted flow cell. For column calibration, the elution volume of tetramer (V_4) was determined using hemoglobin crosslinked between the β-chains with 3,5 (dibromosalicyl)-fumarate [12] and the elution volume of monomer (V_1) using sperm whale myoglobin (Sigma). The elution volume of the hemoglobin dimer (V_2) was then calculated assuming a linear relationship between elution volume and the logarithm of molecular weight.

3. Results

The experiments were designed to measure small differences in the dimer–tetramer association constants of recombinant hemoglobins with respect to natural HbA, in order to determine the effect of specific amino acid substitutions on the dimer–tetramer association. A differential technique has been employed which involves the layering of a natural HbA solution over a recombinant hemoglobin solution at the same protein concentration [6]. In these experiments small differences in dissociation behavior can be detected easily since the presence of a transitory increase or a transitory decrease in absorbance at the

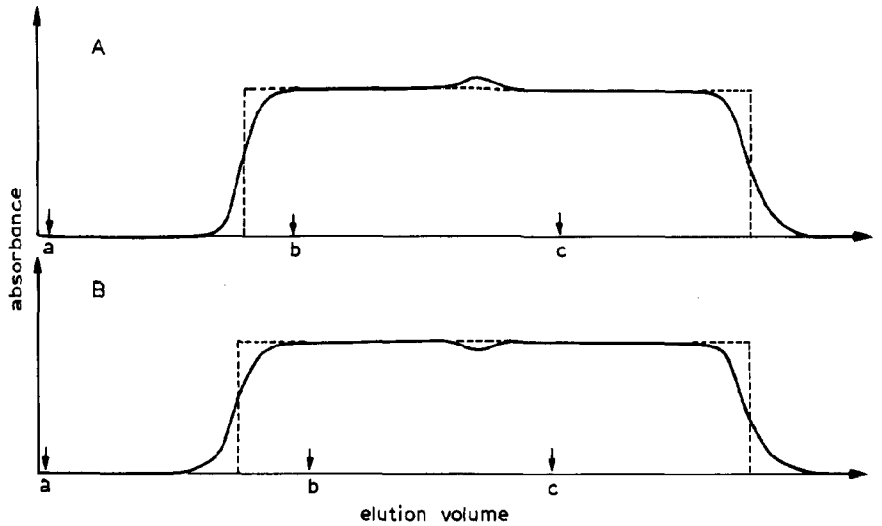

Fig. 1. Differential gel filtration experiments of carbonmonoxy β(Val1 → Met + His2 → del) (A) and β(Cys112 → Gly) (B) versus natural carbonmonoxy HbA. Protein concentration, 0.025 (A) and 0.016 mg/ml (B) in CO saturated sodium tetraborate-NaCl-phosphate-EDTA buffer at pH 7.0, temperature 8.5°C. Arrows indicate the application of: (a) the recombinant protein solution, (b) the HbA solution and (c) buffer. The areas in (A) and (B) correspond to an elution volume difference, ΔV, of 0.34 and 0.26 ml, respectively. In turn ΔV is related to the degree of dissociation into dimers, α, by the expression $\Delta V = (V_2 - V_4)(\alpha_{HbA} + \alpha_{Hb\text{-}Mutant})$.

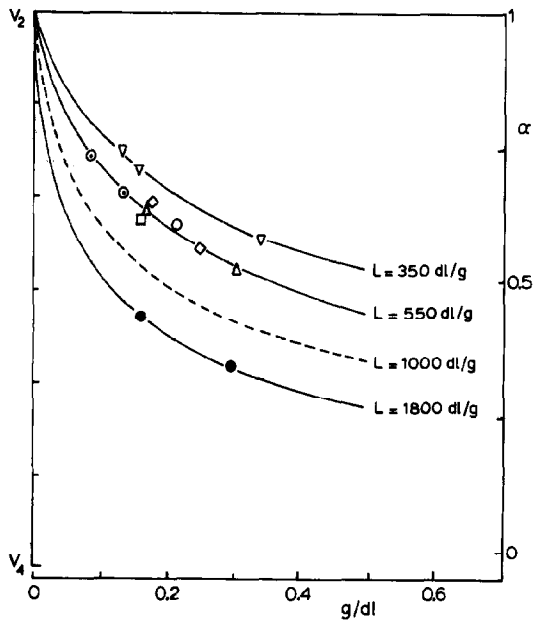

Fig. 2. Elution volume as a function of the concentration of recombinant and native hemoglobin in the carbonmonoxy form. The values of α represent the fraction of dimers present in solution. Buffer: CO saturated 10 mM sodium tetraborate–100 mM NaCl–10 mM phosphate – 1 mM EDTA pH 7.0. Temperature 8.5°C. Recombinant hemoglobins: (□) HbA-Wt; (△) β(Cys93 → Ala); (○) β(Val67 → Thr); (▽) β(Thr4 → Asp); (◇) β(Val1 → Met + His2 → del); (●) β(Cys112 → Gly). The curves were drawn on the basis of indicated dimer–tetramer association constants; the dashed curve represents the behavior of native HbA.

junction of the two hemoglobin solutions is indicative of a difference in their elution volumes (Figs. 1A and 1B). In turn, the area enclosed by the discontinuity is directly related to the difference between the association constants of the two solutions [7].

Initially, the dimer–tetramer association constant of carbonmonoxy HbA was determined to be 1000 dl/g by means of frontal gel filtration experiments [11]. Since all other measurements were of a differential kind, this value is taken as the reference point.

In the differential experiments with HbA-Wt, β(Val67 → Thr), β(Thr4 → Asp), β(Val1 → Met + His2 → deleted), a small transitory increase in the absorbance of the elution profile was observed, indicating a slightly larger dissociation of these recombinant tetrameric hemoglobins with respect to natural HbA. The elution profile of β(Cys93 → Ala) indicates a small increase in dissociation with respect to HbA-Wt. In contrast, the elution profile of β(Cys112 → Gly) presents a transitory decrease in the absorbance indicative of increased stabilization of the carbonmonoxy hemoglobin tetramer with respect to natural HbA. Representative elution profiles are shown in Fig. 1.

The experimental results are reported in Fig. 2 in terms of elution volume and degree of dissociation as a function of protein concentration. The dimer–tetramer association constants are summarized in Table 1 along with the free energies of association.

Table 1
Comparison of dimer-tetramer association constants for natural, recombinant wild-type, and mutant hemoglobins [a]

Hemoglobins	$L_{2,4}$ (dl/g)	K [b] (M^{-1})	ΔG (kcal/mol)
HbA	1000	16.0×10^5	7.97
HbA-Wt	550	8.8×10^5	7.63
β(Thr4 → Asp)	550	8.8×10^5	7.63
β(Val67 → Thr)	550	8.8×10^5	7.63
β(Val1Met + His2del)	550	8.8×10^5	7.63
β(Cys93 → Ala)	350	5.6×10^5	7.38
β(Cys112 → Gly)	1800	28.0×10^5	8.28

[a] Conditions: 10 mM phosphate, 10 mM borate, 100 mM NaCl, pH 7.4 at 8°C.
[b] MW = 32000.

4. Discussion

Assembly of subunits into multimeric proteins depends upon proper folding of the subunits. It follows that equilibrium constants associated with the assembly process are a sensitive measure of conformational abnormality in the subunits, whether they arise from incorrect folding or from conformational changes due to amino acid substitutions. In the case of hemoglobin, the alignment of groups at the $\alpha_1\beta_2$ interface is critical to the functional properties of hemoglobin, since it is this interface that slides in the transition between the two major quaternary conformational states [5].

HbA-Wt has a two fold smaller dimer–tetramer association constant than natural HbA. Thus, at a concentration of 0.5 mg/ml, 35% of natural MbA is dissociated into dimers as compared to 43% in HbA-Wt (Fig. 2). The difference in the degree of dissociation becomes vanishingly small at protein concentrations above 1 mg/ml. This difference is energeticaly very small, indicating that recombinant β-chains correctly refold to yield tetrameric hemoglobin. Similar evidence has been obtained for recombinant hemoglobins reconstituted from β-chains obtained with a different expression system [8]. Any further difference observed in the mutant proteins may be taken to reflect specific effects of the amino acid substitutions. Mutations on the surface of the molecule, β(Thr4 → Asp), in the heme pocket, β(Val67 → Thr), and at the 2,3-DPG binding site, β(Val1 → Met + H2 → del), appear to have little effect on the stability of the carbonmonoxy liganded tetramer, as these mutants have the same association constant of HbA-Wt (Table 1 and Fig. 2). These substitutions are far removed from the $\alpha_1\beta_2$ interface and would not be expected to perturb the dimer–tetramer association. The mutation β(Cys93 → Gly) at the $\alpha_1\beta_2$ interface slightly enhances dissociation, consistent with data on another hemoglobin derivatized at this residue [13]. Unexpectedly the substitution β(Cys112 → Gly) at the non dissociable $\alpha_1\beta_1$ interface results in a two to four fold stabilization of the tetramer in comparison to natural and recombinant HbA, respectively. This result is particularly interesting since it indicates that structural changes at the $\alpha_1\beta_1$ interface can be propogated to the $\alpha_1\beta_2$ interface; in turn it suggests that the $\alpha_1\beta_1$ interface participates in the modulation of the allosteric equilibrium in hemoglobin.

Acknowledgement

This study was supported by PHS NIH grants HLBI-13164 (CF), GMS-35132 (AL) and POI-HL48517 (CF, AL); by 40% and 60% grants of the Ministero Università Ricerca Scientifica. (EC) and by the Research Incentive Fund, Temple University (WSB).

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