Refolding Defects in Hemoglobin Rothschild*

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The substitution of tryptophan by arginine at position 37 within the C helix of the β chain in hemoglobin (Hb) Rothschild (R) (Gacon, G., Belkhodja, O., Wajeman, H., Labie, D., and Najman, A. (1977) FEBs Lett. 22, 243-246) greatly enhances tetramer to dimer association in the liganded form by virtue of this alteration in the αβ contact region. In addition, major changes in the quaternary structure and the folding of the isolated βR subunit occur. At concentrations such that βR is tetrameric, βR is monomeric. Moreover, whereas the far ultraviolet circular dichroism spectra, indicative of backbone conformation, are comparable for HbA and HbAt, suggesting similar folding of chains within these hemoglobins, the isolated βR chain exhibits a reduced far UV CD intensity by comparison with βA.

Like the hemoglobins themselves, the respective apohemoglobins derived from them are similar to each other in secondary and quaternary structure, both being predominantly dimeric with identical far UV CD spectra. In the near ultraviolet regions, the CD differences may be ascribed solely to the absence of the substituted tryptophan residue. The difference CD spectrum in the near UV thus represents the CD of tryptophan 37β in apohbA.

Both apohbR and βR globin show defects in refolding. Thus while the stoichiometric binding of dicyanohem to both apohbR and βR globin restores the Soret band at 405 nm, it does not lead to recovery of the native structures of HbR and βR, in contrast to the complete refolding that occurs with apohbA and βA globin. Moreover, the lack of the characteristic alloplex reaction (Waks, M., Yip, Y. K., and Beychok, S. (1973) J. Biol. Chem. 248, 6462-6470) between the αα heme-containing chain and the βR globin is further evidence of refolding defects. Finally, the substitution also appears to affect correct folding in more distant portions of the mutant β chain as well.

In human hemoglobin, the αβ interface comprises 19 residues (about 34 atoms), with critical contacts between the C helix of each chain and the FG corner of the other (1).

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variants with substitutions in this interface are likely to exhibit both structural and functional alterations because of the intimate involvement of residues in that interface with the mechanism of heme-heme interaction and the stability of the tetrameric structure (for recent reviews, see Baldwin and Chothia (1) and Perutz (2)). When such substitutions are in the β chain, they may also affect the stability of analogous interfaces that occur in the isolated βA chains (3), which form tetramers (HbH). Furthermore, since the αβ interface is disrupted when heme is removed, substitution of contact residues at the interface may influence the structure of apohemoglobin, as well as the refolding that occurs when heme is added to apohemoglobin or to the separated, heme-free mutant chain (4).

We have recently been examining conformational effects brought by a substitution at position 37 in the C helix of the β chain, as occurs in Hb Rothschild (TrpC-3(37)β → Arg). Another hemoglobin with a substitution at this position, Hb Hirose (TrpC-3(37)β → Ser), is also known and has been studied in some detail. Hb Hirose has been shown to be largely dimeric in the carbonmonoxide and deoxy forms under conditions where liganded hemoglobin A is mainly tetrameric. This mutant shows low cooperativity and high affinity for oxygen (5). The functional properties of Hb Rothschild appear to be affected differently, since the latter shows decreased oxygen affinity as well as a low Hill coefficient (6).

The present study was undertaken to determine the effect of the Trp → Arg substitution on the quaternary structure and on the folding of Hb Rothschild, as well as on the structure of the isolated β chains in which the substitution occurs. Since it has been suggested that the C helix has marginal tendency to be helical and that these residues may not be helical in the globins (7), it was interesting also to study the consequences of the substitution on the apohemoglobin itself. Finally a tentative reconstitution of Hb Rothschild was undertaken after addition to apohbR of stoichiometric amounts of heme. The alloplex effect (4), which refers to refolding of the disordered isolated globin chains upon combination with the complementary heme chains, was also investigated using the βR globin chain in the presence of the heme-containing α chain. In these studies, Hb Rothschild showed major refolding defects.

EXPERIMENTAL PROCEDURES

Preparation of Hemoglobins—HbR, purified according to Gacon et al. (6) was a generous gift of Drs. Gacon, Wajeman, and Labie. HbA apohemoglobin A and R and βR globin chains were prepared according to the procedures described by Yip et al. (8). For HbR,

† The abbreviations used are: βA and βR, isolated β chains of HbA and HbR, respectively; HbA, Hemoglobin A; HbR, Hemoglobin Rothschild; apohbR, apohemoglobin.
smaller amounts of protein being involved, the separation procedure into chains was adjusted accordingly. The size of the P2 column for buffer equilibration was 2.5 × 35 cm and a column of Whatman CM52 (20 × 1.5 cm) was used for isolation of βR chains. The removal of heme was made under standard conditions (8).

Optical Measurements—All protein solutions were filtered through 0.45 μ Millipore filters before scanning. Absorption spectra were recorded on a Cary 118 spectrophotometer at 4°C. Hemoglobin concentration was measured at 540 nm in the cyanomet form using a molar extinction coefficient of 11 × 10³. Extinction coefficients at 280 nm of apoHbR and β globin R have been calculated on the basis of apoHbA taking into account the substitution of one tryptophan residue. Since in HbR, α and β chains have the same aromatic content, the value used for both separated globin chains, and for the apoHbR, is the same, 10 × 10³ (9).

Circular dichroism measurements were performed at 4°C on a Cary model 60 spectropolarimeter equipped with 6001 CD attachment. Ellipticity values are presented on a mean residue basis, in degrees cm² decimole⁻¹. The mean residue weights are 103 for apoHb and 110 for β globin chains. Solvent baselines were run for each sample; all the spectra are the average of several runs.

Sedimentation—Sedimentation velocity experiments were performed in a Spinco model E analytical centrifuge equipped with an automatic photoelectric scanner. The heme-free globin chains were run at 4°C and scanned at 290 nm. The heme proteins in the cyanomet form were run at the same temperature and scanned at 420 nm.

Determination of Sulfhydryl Reactivity—The Ellman method was used for determining the number of reactive —SH groups of globins as described by Yip et al. (8) in the presence of 20% sodium dodecyl sulfate. 5,5'-Dithiobis(2-nitrobenzoic acid) was obtained from Calbiochem.

Heme Titrations—Hem (type I, crystalline, Sigma) was dissolved in 0.1 n NaOH. The concentration was determined at 385 nm using 5 × 10³ for the molar extinction coefficient (10). Hemin in the cyanomet form was added by small increments to the protein in 0.1 M phosphate, pH 5.9, and the solution was scanned in the Soret (420 nm) after 15 min.

RESULTS

Subunit Dissociation—Hemoglobin Rothschild was sedimented and compared to HbA at a concentration of 2.3 × 10⁻⁵ M (in heme). Table I shows that at this concentration the sedimentation coefficient of HbA is 4.10 S whereas that of HbR is 2.99 S. The βR heme chain was also compared to βA chain. The sedimentation coefficient of βR varies from 1.91 S to 2.39 S in a concentration range of protein from 2.39 × 10⁻⁵ to 8.77 × 10⁻⁵ M. At a lower concentration, 1.83 × 10⁻⁵ M, βA heme chain, which is known to be in a dimer-tetramer equilibrium, has a sedimentation coefficient of 3.88 S. These results can be compared to the sedimentation coefficient of the eA heme chain, which is monomeric and has a sedimentation coefficient of 1.83 S.

The globin chains obtained from HbR were also sedimented. At a concentration of 3.45 × 10⁻⁵ M, the sedimentation coefficient of apoHbR is identical with that of apoHbA at pH 6.7, i.e. 2.65. At pH 5.7, βR globin displays a sedimentation coefficient of 3.0, compared to the value of 2.7 obtained for βA globin at the same concentration of 3.0 × 10⁻⁵ M.

Optical Spectra—Visible absorption spectra of HbR and HbA measured in the 400 to 700 nm range show absorption bands of the same intensity with extrema at the same wavelengths. In the near UV range of 260 to 350 nm, differences in the absorption intensities are observed. Fig. 1 shows a difference spectrum between oxyHbA and oxyHbR. Protein concentration of the samples was adjusted to give the same absorbance at the 540 nm peak of the cyanomet form. The spectrum is typically a tryptophan absorption spectrum with an extremum at 282 nm and two minor absorption bands at 290 and 272 nm. The molar difference extinction at 282 nm is 4.5 × 10³ whereas the molar extinction of a solution of N-acetyl tryptophamide at pH 6.7 is 5.5 × 10³.

ApoHbR and βR globin were titrated with a solution of dCN hemin in 0.1 M NaOH. Fig. 2 shows two titration curves. There is a change of slope between 1.0 and 1.1 hemeq. The reconstituted protein exhibits a Soret absorption maximum at 418 nm and a molar extinction of 1.19 × 10⁶, identical with that of HbA (4).

—SH Titrations—βR heme chain was titrated with 5,5'-dithiobis(2-nitrobenzoic acid) and an average of 2.3 —SH was found per chain. ApoHbR was titrated in the same conditions but after addition of 20% sodium dodecyl sulfate. A value of 1.0 ± 0.2 was consistently found.

Circular Dichroism—The far UV CD spectrum of HbR was measured and compared to the spectrum of HbA in the same conditions, pH 7.0, 0.05 M phosphate. Both spectra yield an identical shape with negative minima at 207 and 222 nm and an ellipticity of ~21 × 10⁴ deg·cm²·decimole⁻¹ at the extremum (222 nm). The βR heme chain, on the contrary, showed a lower ellipticity, the value for [θ]₂₂₂ being ~18 × 10⁴ deg·cm²·decimole⁻¹ as compared to ~21 × 10⁴ for βA.

With respect to the apohemoglobinins obtained from HbA and HbR, the CD spectra in the far UV are similar. Fig. 3 shows the actual far UV CD of apoHbR, with an ellipticity of ~17 × 10⁴ deg·cm²·decimole⁻¹ at the extremum 222 nm.

The magnitude of the ellipticity found in βR globin is, in contrast, greatly diminished in the far UV, compared to that in βA globin; as shown in Fig. 3, the residue ellipticity at 222

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**TABLE I**

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<th>Sedimentation coefficients of hemoglobin and globin chains</th>
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* From Yip et al. (9).

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Dr. J. L. Luchins, personal communication.
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Fig. 2. Recovery of Soret absorbance intensity as a function of added hemin dicyanide. Globin solutions in 0.1 M phosphate, pH 7.0, (2.0 ml) were titrated by adding 7-μl increments of hemin dicyanide solution. Concentration of HbR (C), 1.0 × 10⁻⁵ M; βR globin (◊), 1.0 × 10⁻⁵ M; hemin dicyanide, 3.6 × 10⁻⁴ M. Titration was carried out at 4°C.

nm is −7000°. Thus, removal of heme from βR leads to a reduction in magnitude of 11,000°, as compared to a loss of 4,000 degrees·cm²/decimole observed when βA is freed of heme.

Fig. 4a compares the near UV CD spectra of apoHbA and apoHbR. Both spectra exhibit negative bands at 291, 283, and 271 nm. There is also a shoulder at 257 nm and a large positive band at 252 nm. The intensities of apoHbR are, however, shifted to more negative values. At the extremum (283 nm) the ellipticity of apoHbA is −70 deg·cm²/decimole whereas the ellipticity of apoHbR is −80°. This also holds for the maximum at 252 nm where apoHbA has an ellipticity of 75°C, and apoHbR only 35 deg·cm²/decimole.

Fig. 4b shows the calculated difference CD spectrum between apoHbA and apoHbR globins. The spectrum is characterized by a broad band between 295 and 260 nm with a maximum at 265 nm and another band with a maximum at 252 nm and an ellipticity of 40 deg·cm²/decimole. There is also a shoulder at 258 nm.

Fig. 5 compares the near UV CD of βA globin and βR globin. The spectrum of βR globin is entirely negative in this region. However, although the intensities at 285 nm are very similar (about −80 degrees·cm²/decimole), the difference at shorter wavelengths between the spectra cannot be attributed solely to the absence of the tryptophan residue, as in Fig. 4, since the spectra differ by more than twice the magnitude of the tryptophan contribution shown in Fig. 4b. It should be noted, however, that the far UV spectra of apoHbA and apoHbR are the same, suggesting that the near UV differences arise from differences in aromatic content rather than from...
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**Discussion**

The functional modifications of HbR brought about by the single substitution of arginine for tryptophan at position β37 are accompanied by several structural changes. 1. The quaternary structure of HbR is sharply displaced toward the dimer by comparison with HbA at comparable concentrations. 2. The βR heme subunit, itself, is also altered in quaternary structure and occurs in a monomeric state at concentrations where βA is tetrameric. 3. The isolated βR subunit shows diminished secondary structure as compared to βA. 4. The isolated, heme-free βR globin chain is more disordered than the βA globin chain under the same conditions, whereas apoHbR is not; and 5. despite heme binding and recovery of Soret spectra, neither the βR globin nor the apoHbR recover the structures of the corresponding heme-containing forms when heme is added, in contrast to the globins derived from HbA. With respect to the quaternary structure of HbR, we have not systematically measured the dissociation constant, but from several ultracentrifugation measurements we estimate Kd as about 2.5 × 10^-4 M or about two orders of magnitude greater than that of HbA in liganded states (12).

It might have been supposed that the presence of the charged arginine residue at the aβ interface causes electrostatic repulsions which lead to destabilization of the tetramer. However, two arguments may be used against a predominantly coulombic effect. The first is that Hb Hirose also has a much greater dissociation tendency than HbA, and in that case the substitution is by the neutral serine. The second is that scrutiny of the three-dimensional structure in the vicinity of this position based on recent coordinates (1) reveals the possibility of favorable electrostatic interactions with a neighboring aspartate residue and little opportunity for strong repulsive forces.

It is, therefore, more likely that destabilization of the aβ interface in both HbR and Hb Hirose results from interruption of a pattern of hydrophobic residues which is required for correct folding and stability of this interface. In addition, the requirement for hydration of the charged arginine in HbR may force greater exposure to solvent than occurs in HbA at the corresponding site within the aβ interface.

The reason for the occurrence of the highly unusual monomeric state of βR chain may have some similarities to the foregoing. Arnoff has recently reported the heme-heme distances in the tetrameric βA and shown that all are very similar to those in methemoglobin A (9). This suggests that there may be corresponding interfaces in HbA and in tetrameric βA and that two of the β subunits adopt the spatial orientation, as it were, of two a subunits in HbA. Indeed, there is substantial homology between a and β chains in both contact regions of the aβ interface. Therefore, it is plausible that a residue substitution which disrupts the aβ interface in HbR may also destabilize the similar interface in βR. Here, however, electrostatic effects cannot be excluded because the details of the interface are not known and because the quaternary structure of Hb Hirose has not been reported. Of course, the complementarity in the βA interfaces does not match the precision in HbA, as evidenced by a greater dissociation constant in βA (13). Moreover, the sulfhydril reactivity of βA is different from HbA as shown by Arnoff and Briley (3); in βA, Cys 112 is more reactive than Cys 93.

The underlying mechanisms to account for the refolding defects in apoHbR and in βR globin are also obscure; and we shall, therefore, restrict our discussion of these results to a few observations. To begin with, it is well known that removal of heme from HbA destabilizes the aβ interface, since apoHbA is predominantly dimeric, near neutral pH, and at most accessible concentrations. Moreover, apoHbA is slightly less or-
tered, in secondary structure, than HbA (11). Reconstitution with stoichiometric amounts of heme leads to reformation of the αβ interface and refolding of the unfolded segments. In view of the fact that refolding and re-establishment of the αβ interface are coupled in HbA, the question arises as to whether these two phenomena are obligatorily linked in hemoglobin. The present results with HbR suggest that this may, indeed, be the case. Although heme is stoichiometrically bound to apoHbR, with recovery of characteristic spectral features, the native secondary structure is not recovered. It should be noted that heme is being bound in this experiment to both αA and βR globin chains within apoHbR but neither chain refolds, suggesting further that the refolding of both chains is dependent on the reformation of the correctly folded αβ interface.

Evidently, the refolding defect observed here is overcome in vivo, since the native HbR has the same CD in the far UV as HbA, suggesting that the correct folding depends on a particular order of addition, or on precise conditions of concentration, pH, or other conditions which are not duplicated in vitro.

Finally, we wish to note that there are two further folding anomalies in this system connected with the mutant β globin. The first is that βR globin is much more disordered than βA globin under the same conditions. The second is that βR globin is not refolded either by direct heme addition or by addition of the complementary α heme-containing subunit. Again, both of these latter defects may be linked to a failure to form necessary interfaces which are themselves correctly folded.

In the case of direct heme addition, the stability of the refolded form may depend on solvent exclusion and hydrophobic bonds in the tetrameric state. The failure to observe any structural change when αA heme chain and βR globin are mixed may be a reflection of the fact that the substitution can affect refolding in more distant portions of the chain.

Because of the far reaching structural and functional changes resulting from the substitution, HbR may be very useful in investigations of various other reactions of hemoglobin, such as its specific binding to haptoglobin. Rogard and Waks have shown that a hemoglobin tryptophan residue becomes inaccessible upon complex formation with haptoglobin and have suggested that tryptophan 37β is the most likely candidate (14). Studies with HbR should help establish whether this tentative identification is correct and will shed light on the nature of the intermolecular contacts in the complex.

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REFERENCES