Recombinant Human Hemoglobin: Modification of the Polarity of the β-Heme Pocket by a Valine^{67}(E11) → Threonine Mutation†

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ABSTRACT: Using the mutagenesis and a gene expression system previously described [Fronticelli et al. (1991) *J. Protein Chem. 10*, 495–501], we have replaced Val^{67}E11 in the distal heme pocket of the β-chains of hemoglobin with Thr. The valine to threonine substitution is isosteric and only modifies the polarity of the β-heme environment. The absorption and CD spectra of the resultant mutant hemoglobin were essentially the same as that of wild-type protein, indicating that the mutation did not cause any large conformational changes and that a water molecule was not coordinated to the ferrous iron atom. Equilibrium measurements of oxygen binding to the mutant indicate a 2-fold decrease in overall affinity relative to native or wild-type human hemoglobin. Thermodynamic analyses of O_2 binding curves, based either on the sequential Adair model or on the MWC two-state model, indicated that the overall decrease of O_2 affinity in the system was due to a lower association equilibrium constant for the intermediates of oxygenation, particularly those involved at the third ligation step. The functional characteristics of the mutant hemoglobin in either the T- or R-state were not modified greatly by the mutation; however, the Bohr effect and sensitivity to Cl^- were increased, suggesting a role of the intermediates of oxygenation in the modulation of these parameters. Kinetic measurements of the last step ($Hb_4X_3 + X \rightarrow Hb_4X_4$) in ligand binding showed that the βVal^{67}(E11) → Thr mutation decreases the O_2 association rate constant roughly 2-fold, has no effect on the O_2 dissociation constant, has no effect on the CO association rate constant, and increases the CO dissociation rate constant roughly 2-fold. The net result of these effects is a 2-fold decrease in the equilibrium constant for both O_2 and CO binding to β subunits in the triliganded intermediate. Unexpectedly, the βVal^{67}(E11) → Thr mutation caused a 3-fold increase in the rate of CO binding to β-subunits within T-state deoxyhemoglobin.

Access of ligands to the heme group in myoglobin and hemoglobin is hindered by the presence of the globin chain, which buries the heme in a hydrophobic crevice in the protein interior. Iron–ligand combination must therefore be accompanied by conformational fluctuations in the protein which create a pathway for ligand entry. Ligand binding to the heme is believed to be regulated by steric hindrance and polar interactions within the heme pocket. It has been proposed that the pathway of ligand entry in myoglobin involves ligand movement between the side chains of His-E7 and Val-E11 (Case & Karplus, 1979; Kottalam & Case, 1988). Studies on mutant myoglobins and hemoglobins where His-E7 was replaced by a variety of amino acids of different size and polarity indicated that the ligand association and dissociation rate constants are regulated by this residue in sperm whale myoglobin and in R-state[1] α-subunits (Rohlfs et al., 1990; Olson et al., 1988; Mathews et al., 1989). In the R-state β-subunits, replacement of His-E7 had a much smaller effect, indicating that the distal histidine does not play a major role in the modulation of ligand binding in this form of the β-heme pocket.

The role of Val-E11 has been investigated in sperm whale myoglobin (Egeberg et al., 1990) and in the α- and β-subunits of human hemoglobin (Nagai et al., 1987; Olson et al., 1988; Mathews et al., 1989; Tame et al., 1991) by replacing this residue with apolar amino acids of different sizes. Increasing the size of the αE11 side chain from Ala to Val to Leu to Ile did not appear to modify the oxygen affinity of α-subunits in either quaternary state (Tame et al., 1991). These substitutions, however, did modify the association and dissociation rate constants for the O_2 binding to R-state α-subunits (Mathews et al., 1989) and myoglobin (Egeberg et al., 1990), indicating that this residue is part of the dynamic barrier which controls access to the iron atom. In β-subunits, replacement of Val^{67}E11 with Ala caused an increase in T-state O_2 affinity with little or no change in R-state affinity (Nagai et al., 1987; Mathews et al., 1989). Replacement of βVal^{67}-E11 with Ile produced a right shift of the entire equilibrium binding curve, indicating a substantial decrease in the oxygen affinity of both quaternary states. In this latter mutant, the C_δ atom of the side chain hinders access of the ligand to the heme iron atom (Nagai et al., 1987). Kinetic measurements

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[1] Abbreviations: HbA_0, normal human hemoglobin; Hb-βV^{67}(E11)T, mutant hemoglobin in which Val^{67}(E11) of β-chains was replaced by Thr; R-state, oxy conformation of hemoglobin; T-state, deoxy conformation of hemoglobin; FX, factor Xa recognition sequence; RF, replicative form; dNTPs, deoxynucleoside triphosphates; DTT, dithiothreitol; LB, Luria broth, SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DEAE, diethylaminoethyl.

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were consistent with the equilibrium measurements for βIle^{67}-E11. These data showed that Ile at position βE11 produces a substantial decrease in the observed association rate constant for binding to both R- and T-states (Mathews et al., 1989, 1991). All of these previous data suggest that Val^{67}E11 may be the key residue in regulating ligand binding to β-subunits.

We have investigated the effect of a polar substitution at E11 by replacing β-Val^{67}E11 with Thr. Since the two amino acids are isosteric and have a similar molecular volume, this mutation only modifies the polarity of the environment, simplifying interpretations. Equilibrium and kinetic measurements were carried out to determine the oxygen binding parameters of Hb-βV^{67}(E11)T for the stepwise addition of O_2 to the mutant tetramer. In a previous paper, we demonstrated that the conformational and functional characteristics of HbA_0 and wild-type Hb are very similar (Fronticelli et al., 1991).

Smerdon et al. (1991) constructed the Val(E11) → Thr substitution in pig myoglobin and observed a marked 15-fold decrease in O_2 affinity. In contrast, increasing the polarity of the β pocket with Thr^{67}(E11) produces only a 2-fold decrease in the O_2 affinity of human hemoglobin. This effect is due primarily to modifications of the properties of the intermediates of oxygenation and not to changes in the intrinsic ligand binding parameters of the initial T- and final R-state.

MATERIALS AND METHODS

Mutagenesis. The β-globin expression system routinely used is pJKO5 in *Escherichia coli* strain AR120. This plasmid contains the human β-globin cDNA fused to 243 nucleotide residues of the NS1 influenza virus gene. A factor Xa recognition sequence (FX) separates the truncated NS1 gene and β-globin in a manner strictly analogous to pLcIIFXβ-globin of Nagai and Thogersen (1984). Construction of pJKO5 has been previously reported (Fronticelli et al., 1991). All routine molecular cloning techniques were carried out as described by Maniatis et al. (1982). The Val codon, GTG, coding for amino acid residue 67 in the human β-globin cDNA was changed to the Thr codon, ACG, using the M13 system as described by Zoller and Smith (1984). The 934-bp *Hin*dIII fragment from pJKO5, containing the entire coding sequence for NS1–FX–β-globin, was inserted into M13mp11 which had been linearized with *Hin*dIII. Orientation of the insert was determined by hydrolysis with *Eco*RI since there are *Eco*RI sites in both the vector and the insert. A plasmid was selected which produced the antisense strand of β-globin in the single-stranded phase particles. The mutagenic primer, 5′-GGCAAGAAA*ACG*CTCGGTGCC-3′, was synthesized with the sense sequence. Two hundred picomoles of this nucleotide, to be used as the mutagenic primer, was phosphorylated on the 5′-end with ATP and T4 polynucleotide kinase. Using the same reaction, 20 pmol of the oligonucleotide, to be employed later for plaque hybridization, was phosphorylated with [γ-^{32}P]ATP (3000 Ci/mmol). Separation of the labeled oligonucleotide from unreacted ATP was accomplished by passage of the reaction mixture through a Sephadex G25 column and elution with 10 mM Tris, 1 mM EDTA, and 100 mM NaCl, pH 8.0.

Generation of the mutagenized β-globin gene utilized a method adapted from Kramer et al. (1984) and involved first the formation of a gapped heteroduplex between 0.5 pmol of the replicative form (RF) of M13mp11 and 0.75 pmol of single-stranded M13mp11 into which the NS1–FX–β-globin DNA had been inserted as described above. The two were mixed in a volume of 11 μL of 100 mM Tris, 0.6 M NaCl, and 66 mM $MgCl_2$, pH 8.0. The solution was heated at 100 °C for 5 min and slowly cooled to 65 °C over a period of 15 min. Twenty picomoles of the phosphorylated mutagenic primer was added and the temperature was maintained at 65 °C for 10 min. The solution was then allowed to cool to room temperature over a period of 30 min. The following additions were made to the solution: 4 μL of 2 mM dNTPs, 1 μL of 20 mM ATP, 1 μL of 100 mM DTT, 2 units of T4 DNA ligase, and 5 units of Klenow fragment. The reaction was allowed to proceed at room temperature for 45 min and then the entire mixture was used to transfect competent JM101 cells. The cells were plated on Luria broth (LB) in top agar and allowed to grow overnight at 37 °C. Plaque hybridization were carried out essentially as described by Benton and Davis (1977), using the ^{32}P-labeled primer as probe. The hybridizations were allowed to equilibrate overnight, and the filters were washed at 23 and 62 °C. A positive plaque was removed from the plate with a sterile Pasteur pipet and used to infect a JM101 culture. The RF form of the plasmid bearing the altered codon was isolated from the bacterial pellet and the single-stranded form was isolated from the supernate. The latter was used for sequencing by the Sanger dideoxy method (Sanger et al., 1977). A deoxyoligonucleotide with the sequence 5′-GGCCCTGGGCAGGCTGC-3′, which is complementary to a region of the β-globin gene near the 5′-end, was used as the sequencing primer. Both the mutant gene and the native gene were sequenced at the same time, running both series of reactions on the same gel. The sequence of the native gel was identical to that originally reported for the exon portion of the human β-globin gene (Lawn et al., 1980), and the only difference between the native and mutant sequences was at the intended site, codon 67 being ACG instead of GTG in native.

The RF form of the plasmid, isolated from the bacterial pellet, was hydrolyzed with *Nco*I and *Cla*I and the 700-bp fragment bearing the factor Xa recognition sequence plus the β-globin gene was isolated. This fragment was inserted into pJKO1 (Fronticelli et al., 1991) which had been linearized with the same two restriction enzymes. The resulting plasmid was identical to PJKO5 except for the two base changes in the globin gene. *E. coli* strain AR120, transformed with this plasmid and induced with nalidixic acid, overproduced a fusion protein of the same mobility in SDS–PAGE as the native fusion protein from pJKO5 and with the same reactivity toward rabbit anti-human Hb antisera.

Protein Expression and Purification. Cells were grown at 37 °C in an LB + ampicillin medium to an OD of 0.600. Nalidixic acid was added (60 μg/mL) and the growth continued for 24 h. An average yield of such preparations is 2 g of cells/L of culture. The cellular components were solubilized as described by Nagai and Thogersen (1987) and the fusion protein was solubilized and reconstituted with α-chains and heme as described previously (Fronticelli et al., 1991). Purification of the mutant Hb was accomplished on a DEAE-Sephacryl column using the gradient described by Abraham et al. (1976); an average of 10 mg of βThr^{67}(E11) mutant was recovered from 2 g of cells. Cyanide was removed by reduction with dithionite in the presence of CO at atmospheric pressure. After dialysis the protein was stored at −80 °C in the CO form. CO was removed by illumination with white light under a stream of humidified oxygen.

Hemoglobin. HbA_0 was prepared from outdated blood obtained from the blood bank of the University of Maryland (Fronticelli et al., 1991). It could be stored at −80 °C for

several months without detectable methemoglobin formation.

The α-chains were prepared from carbonmonoxy HbA_0 by reaction with *p*-mercuribenzoate (Bucci & Fronticelli, 1965). *p*-Mercuribenzoate was removed from the isolated α-chains (Geraci et al., 1969) and the solution, at a concentration around 10 mg/mL, was stored at −80 °C where the α-chains were stable to oxidation for several months.

Cyanohemin was prepared from protohemin chloride (Midcentury) by first dissolving a few milligrams in 2 mL of 100 mM NaOH. The hematin concentration was determined spectrophotometrically using a molar extinction coefficient at 385 nm of 5×10^4. The solution was diluted with 100 mM phosphate buffer, pH 7.2, to a hematin concentration of 0.4 mg/mL and converted to the cyano complex by addition of twice the stoichiometric amount of KCN. This solution was stored in the cold and used within a week.

Factor X was prepared from bovine plasma (Fujikawa et al., 1972). The proenzyme concentration was measured at 280 nm using $E_{1\%} = 12.4$. The proenzyme (20 mL at 1 mg/mL) in 20 mM Tris buffer at pH 8.0 and 1 mM $CaCl_2$ was activated by recycling in the cold for 6 h through a 1- × 3-cm column of cyanogen bromide activated Sepharose 4B (Pharmacia) coupled to Russell's viper venom. The specific activity of the enzyme was measured as described by Nagai and Thogersen (1987). Alternatively, human factor Xa was obtained from Dr. Walter Kisiel, Department of Pathology, University of New Mexico.

Protein Concentration. For the heme proteins, concentration was measured spectrophotometrically using an absorption coefficient $E_{1\%} = 0.868$ for the carbonmonoxy derivative at 540 nm and a molar extinction coefficient of 11 000 for the cyanomet derivatives. For the non-heme proteins, concentration was measured using the BCA protein assay reagent from Pierce.

Kinetic Measurements. Rate constants for the last step in ligand binding to native and mutant hemoglobins ($Hb_4X_3 + X \rightleftarrows Hb_4X_4$) were determined exactly as described by Mathews et al. (1989). O_2 dissociation rate constants were determined by analyzing time courses for the displacement of bound oxygen by carbon monoxide, and CO dissociation rate constants were determined by measuring the rate of displacement of carbon monoxide by nitric oxide. These rapid mixing experiments were performed using a Gibson-Dionex stopped-flow spectrometer interfaced to an OLIS 3820 data collection system. CO association rate constants for the last step in ligand binding were measured by partially photolyzing fully saturated samples so the extent of breakdown was less than or equal to 10% (i.e., after photolysis the fractional saturation changed from ≥0.90 to 1.00). These experiments were carried out in a conventional flash apparatus consisting of photographic strobes (Sunpack Auto 544) equipped with thyristor quenching devices and a spectrometer system interfaced to another OLIS data collection system. For these experiments, multiple (>10) traces were collected and averaged for each sample. The rates of O_2 rebinding to $Hb_4(O_2)_3$ were measured with the Phase-R 2100B dye laser system described previously (Mathews et al., 1989). All the photolysis traces were fitted to a two-exponential expression with equal amplitudes, and the fast and slow components were initially assigned to the α- and β-subunits by assuming that the native, R-state α-subunit in the mutant tetramer has rate parameters identical to those obtained for native HbA_0.

The T-state CO association parameters were estimated by analyzing time courses for the reaction of excess CO with deoxyhemoglobin samples in a stopped-flow rapid mixing apparatus. These time courses were either accelerating or monophasic and were fitted to a single-exponential expression. Estimation of individual α- and β-subunit rate constants was carried out as described by Mathews et al. (1991) assuming again that the native α-subunit had unaltered properties in the mutant tetramer.

Oxygen Equilibrium Curves, Data Collection, and Analysis. These measurements were performed using the thin-layer dilution technique (Dollman & Gill, 1978). With this technique the oxyhemoglobin is deoxygenated by stepwise dilution of the equilibrating gas with constant volumes of N_2. The protein concentration was 20–30 mg/mL. The oxygen saturation was followed at 436 nm, where absorbance changes due to methemoglobin formation are minimized; the reducing system (Hayashi et al., 1973) produced artificial decreases in the oxygen affinity and was not used. Formation of methemoglobin was judged by comparing the initial spectrum of the oxygenated sample with the spectrum obtained upon reoxygenation of the hemoglobin at the end of the experiment. In the mutant hemoglobin this last step is associated with methemoglobin formation; thus our estimates are in excess over the actual amount of methemoglobin present in Hb-βV[67](E11)T at the end of the measurements. Formation of methemoglobin was absent in the native HbA_0 samples; however, it was present in the reoxygenated samples of Hb-βV[67](E11)T, in amount which varied between 10 and 20%. Changes in absorbance, upon stepwise changes in oxygen partial pressure, were followed using an AVIV 14DS spectrophotometer. The data were analyzed using the relationship

$$\Delta\theta_i = \Delta\theta_T \Delta Y_i \quad (1)$$

where $\Delta\theta_i$ is the absorbance change at each step of the titration *i*th oxygen, ΔY_i is the change of fractional saturation of hemoglobin with oxygen, and $\Delta\theta_T$ is the total absorbance change obtained in going from deoxy- to oxyhemoglobin [Gill et al. 1987]. These data were fitted to the Adair equation (Adair, 1925) using an iterative procedure incorporating the Marquardt algorithm:

$$Y = \frac{\beta_1 P_{O_2} + 2\beta_2 P_{O_2}^2 + 3\beta_3 P_{O_2}^3 + 4\beta_4 P_{O_2}^4}{4(1 + \beta_1 P_{O_2} + \beta_2 P_{O_2}^2 + \beta_3 P_{O2}^3 + \beta_4 P_{O_2}^4)} \quad (2)$$

where Y is the fractional saturation with oxygen, P_{O_2} is the partial pressure of oxygen in millimeters of mercury, and β_i's are the overall Adair constants related to the intrinsic statistical affinity constants K_i of the subsequent steps of oxygenation by $\beta_i = \prod_i K_i$. The value of the median ligand activity, P_m, was estimated from $P_m = \beta_4^{-0.25}$ (Wyman & Gill, 1990). When all the parameters are allowed to float, the values of β_1 and β_4 had standard errors of 25% and 10%, respectively; however, the values of β_2 and β_3 had standard errors in excess of 100%. To allow better estimation of these constants, we fixed in the analysis the ratio of β_4 to β_3 to the value of the affinity constant $K_4/4$ which was computed from independent kinetic measurements. On the basis of the definition of the overall Adair constant we can write

$$\beta_4 = \beta_3 K_4/4 \quad (3)$$

K_4 was computed from the kinetics of ligand rebinding to the species Hb_4X_3 using

$$K_4 = 2K_\alpha K_\beta/(K_\alpha + K_\beta) \quad (4)$$

where K_α and K_β are the ratios of the on and off rate constants for the individual α- or β-subunits, respectively (see Table IV). The substitution of eq 3 into eq 2 allowed an estimation

Table I: Parameters for the Oxygen Binding Isotherms of HbA_0 and Hb-$\beta V^{67}(E11)T$[a]

	P_m[b]	P_{50}[c]	n[d]	β_1[e] ($Torr^{-1}$)	β_2 ($Torr^{-2}$)	β_3 ($Torr^{-3}$)	β_4 ($Torr^{-4}$)
HbA_0	6.1	6.49	2.6	$(12.3 \pm 3) \times 10^{-2}$	$(16 \pm 2) \times 10^{-3}$	$(6 \pm 0.7) \times 10^{-4}$	$(7.3 \pm 0.6) \times 10^{-4}$
Hb-$\beta V^{67}(E11)T$	10.9	13.9	2.2	$(15.8 \pm 2) \times 10^{-2}$	$(13 \pm 1) \times 10^{-3}$	$(7 \pm 0.9) \times 10^{-5}$	$(7.0 \pm 0.8) \times 10^{-5}$

[a] Parameters were estimated with the Adair equation (eq 2). Measurements were performed at 20 °C, in 100 mM Bis-tris and 100 mM KCl at pH 7.0. [b] P_m = median partial pressure. [c] P_{50} = partial pressure of O_2 far 50% saturation. [d] n = Hill parameter. [e] β_1 = overall equilibrium constant.

of the Adair constants β_2 and β_3 with only a 10% standard error, while the constants β_1 and β_4 remained practically unchanged from the values obtained leaving all the parameters floating (see Table I).

The oxygen equilibrium curves were also analyzed according to the Monod–Wyman–Change (MWC) two-state model:

$$Y = \frac{L_0 K_T P_{O_2}(1 + K_T P_{O_2})^3 + K_R P_{O_2}(1 + K_R P_{O_2})^3}{L_0(1 + K_T P_{O_2})^4 + (1 + K_R P_{O_2})^4} \quad (5)$$

where Y is the fractional saturation of hemoglobin with O_2, L_0 is the conformational equilibrium constant between the T- and R-forms in the absence of ligands, K_T is the affinity constant of the T-form for O_2, and K_R is the affinity constant of the R-form for O_2. As shown by Wyman and Gill (1990) for moderate values of L_0 ($<10^6$) the oxygen equilibrium constant for the fourth molecule of oxygen, K_4, represents the oxygen affinity of R-state Hb, K_R. In the fitting procedure we have fixed K_R to the value of K_4 determined kinetically using eq 4.

The number of protons liberated per mole of heme upon oxygen binding, ΔH^+, was obtained using (Wyman, 1964)

$$\Delta H^+ = -\mathrm{d} \log P_m / \mathrm{d}\, \mathrm{pH} \quad (6)$$

Sedimentation velocity measurements were performed with a Beckman Model E analytical ultracentrifuge, using the schlieren optics. The temperature was between 19 and 21 °C.

Optical measurements were done using an AVIV 14DS spectrophotometer and an AVIV 60DS spectropolarimeter.

RESULTS

Optical Measurements. The isosteric characteristics of the valine and threonine suggest that the conformation of the protein as a whole, and of the heme pocket in particular, should not be disrupted by the mutation. In order to verify this idea we compared the absorption spectra in the visible and Soret regions of HbA_0, wild-type Hb, and Hb-$\beta V^{67}(E11)T$ in the oxy, carboxy, and deoxy forms. The absorption spectra were very similar and shifts in the wavelengths of maximum absorption were not detected.

The CD spectra of Hb-$\beta V^{67}(E11)T$ were compared to those for wild-type hemoglobin (Fronticelli et al., 1991). The spectra were recorded for the cyanomet form to eliminate interference due to methemoglobin formation. The spectra, recorded in the visible, Soret, near UV, and far UV regions of the spectrum, were the same for the two hemoglobins. The CD spectra of the deoxy forms were also recorded in the Soret region in the presence of 1 mg/mL dithionite, and again results for the two hemoglobins were identical.

Sedimentation Velocity. Sedimentation velocity was measured in 0.05 M phosphate buffer at pH 7.15 at a protein concentration of 2.5 mg/mL. It showed a single symmetrical peak with an S_{20w} of 4.22. Natural Hb under the same conditions had a S_{20w} of 4.25. These data confirm that Hb-$\beta V^{67}(E11)T$ is a stable tetramer like natural Hb.

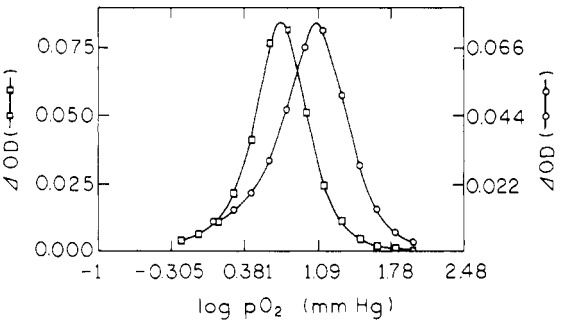

FIGURE 1: Differential oxygen-binding data for HbA_0 (□) and Hb-$\beta V^{67}(E11)T$ (○) vs the logarithm of the partial oxygen pressure. Buffer: 10 mM Bis-tris and 100 mM KCl at pH 7.0. Temperature: 20 °C. The lines represent the best fit to the data obtained with eq 2 and the parameters listed in Table I.

Equilibrium Measurements. The oxygen affinity of Hb-$\beta V^{67}(E11)T$ was 2-fold decreased with respect to HbA_0. Representative binding isotherms expressed as differential absorbance change are shown in Figure 1, where the symbols represent the experimental points obtained and the continous line represents the fit obtained using eq 2 (Adair, 1925). Analyses using eq 5 (Monod et al., 1965) gave similar fits. These measurements were done at 20 °C in 100 mM Bis-tris and 100 mM KCl buffer, conditions identical to those used for the kinetic measurements. In these analyses, K_4 obtained from independent kinetic measurements was used to fix either the ratio β_4/β_3 in the Adair model or K_R in the MWC model, as described in Materials and Methods. The values of K_4, computed with eq 3, were $(3.04 \pm 1.15) \times 10^6\ M^{-1}$ (=4.99 $Torr^{-1}$) for HbA_0 and $(2.39 \pm 0.8) \times 10^6\ M^{-1}$ (=3.92 $Torr^{-1}$) for Hb-$\beta V^{67}(E11)T$. The other fitted parameters are given in Tables I–III.

Inspection, in Table II, of the variation of $\delta\Delta G$ values associated with the four steps of oxygenation K_i's shows that the difference in free energy of oxygenation between HbA_0 and Hb-$\beta V^{67}(E11)T$ is expressed primarily by the third oxygenation step, with a $\delta\Delta G$ of 1.1 kcal/mol. When the analyses were carried out according to the MWC model, a similar $\delta\Delta G$ was computed for the differences between the allosteric constants L_0 of the native and mutant proteins (Table III). These $\delta\Delta G$ values are very similar to those corresponding to the difference in P_{50} between HbA_0 and Hb-$\beta V^{67}(E11)T$ (1.4 kcal/mol). The K_4 values used in these analyses were very similar. When, in both proteins, they were either fixed to the same value ± the standard deviation or interchanged, the $\delta\Delta G$ values between the various parameters of the two systems were not modified significantly.

As shown in Figure 2, the oxygen affinity of Hb-βV^{67}-(E11)T is less than that of HbA_0 at all pH values, the effect being particularly evident below pH 7.2. At pH 6.4 the P_{50} increases from 16 mmHg for HbA_0 to 30 mmHg for Hb-$\beta V^{67}(E11)T$. By using eq 6, it can be calculated that at pH 7.2 the oxygen-linked protons are 2.1 for tetrameric HbA_0 and 2.9 for tetrameric Hb-$\beta V^{67}(E11)T$.

The effect of increasing Cl^- concentration on the oxygen affinity of HbA_0 and Hb-$\beta V^{67}(E11)T$ is shown in Figure 3.

Table II: Comparison between the Parameters Recovered for HbA_0 and Hb-βV[67](E11)T[a]

	HbA_0		Hb-βV[67](E11)T		
i	K_i (Torr^{-1})	ΔG (kcal/mol)	K_i (Torr^{-1})	ΔG (kcal/mol)	HbA_0 – Hb-βV[67](E11)T $\delta\Delta G$ (kcal/mol)
1	$(308 \pm 76) \times 10^{-4}$	−5.9	$(395 \pm 50) \times 10^{-4}$	−6.0	0.1
2	$(867 \pm 320) \times 10^{-4}$	−6.5	$(548 \pm 109) \times 10^{-4}$	−6.2	0.3
3	$(562 \pm 109) \times 10^{-4}$	−6.2	$(81 \pm 14) \times 10^{-4}$	−5.1	−1.1
4	4.99[b]	−8.8	3.92[b]	−8.7	−0.1

[a] Parameters were recovered using the Adair equation (eq 2) where $\beta_4 = \beta_3 K_4/4$ as described in the text. [b] Fixed value in the numerical analyses.

Table III: Comparison between the Parameters Recovered for HbA_0 and Hb-βV[67](E11)T[a]

	HbA_0		Hb-βV[67](E11)T		
	MWC parameters	ΔG (kcal/mol)	MWC parameters	ΔG (kcal/mol)	HbA_0 – Hb-βV[67](E11)T $\delta\Delta G$ (kcal/mol)
L_0	$(77 \pm 2) \times 10^4$	−6.6	$(45 \pm 1) \times 10^5$	−7.6	1.0
K_T (Torr^{-1})	$(45 \pm 2) \times 10^{-3}$	−3.3	$(38 \pm 0) \times 10^{-3}$	−3.4	0.1
K_R (Torr^{-1})	4.99[b]	−8.8	3.92[b]	−8.7	−0.1

[a] Parameters were recovered using the MWC equation (eq 5) with $K_R = K_4$ as described in the text. [b] Fixed value in the numerical analyses.

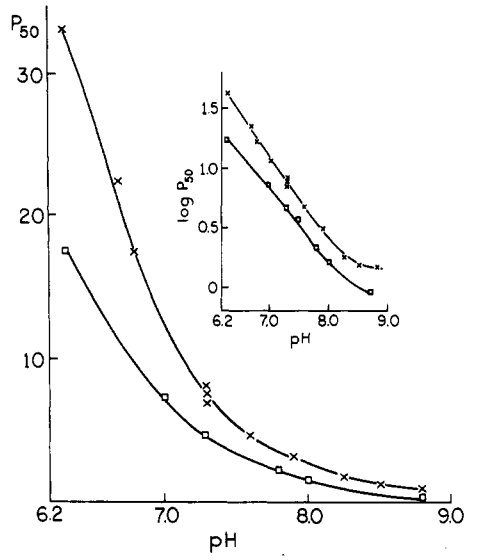

FIGURE 2: pH dependence of oxygen affinity of HbA_0 (□) and Hb-βV[67](E11)T (×). Inset: Logarithm plot of the same. Buffer: 10 mM monobasic phosphate, 10 mM borate, and 100 mM NaCl; the buffer was adjusted at the various pH values with HCl. Temperature: 25 °C.

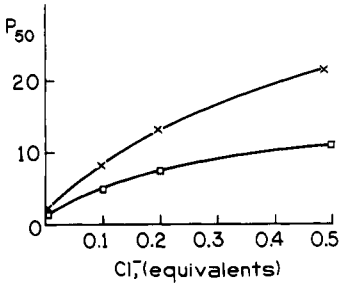

FIGURE 3: Effect of Cl^- ion concentration on the oxygen affinity of HbA_0 (□) and Hb-βV[67](E11)T (×) in 15 mM Hepes buffer. Temperature: 25 °C.

It is evident that sensitivity to Cl^- is more pronounced in Hb-βV[67](E11)T. Using an expression similar to eq 6, it can be calculated that the O_2-linked Cl^- are 1.6 and 2.4 in tetrameric HbA_0 and Hb-βV[67](E11)T, respectively.

Kinetic Analyses of the Last Step in Ligand Binding ($Hb_4X_3 + X \rightarrow Hb_4X_4$): R-State Constants. Representative time courses for O_2 rebinding to partially photolyzed native and

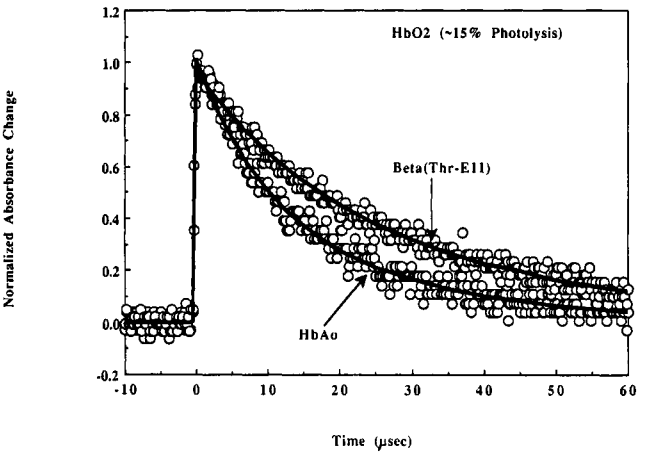

FIGURE 4: Normalized time courses for the recombination of 1.25 mM O_2 with hemoglobin following photolysis by a 300-ns laser flash. The observed time courses were fitted to a two-exponential expression with equal amplitude (solid lines). HbA_0: normal human hemoglobin. β(Thr-E11): mutant hemoglobin in which Val[67](E11) was replaced by Thr. As described elsewhere (Mathews et al., 1989), the faster component in native HbA_0 represents O_2 binding to β-subunits (≈100 μM^{-1} s^{-1}) and the slower component represents binding to α-subunits (≈30 μM^{-1} s^{-1}). Buffer: 10 mM Bis-tris and 100 mM KCl at pH 7.0. Temperature: 20 °C.

mutant hemoglobin are shown in Figure 4. The solid line represents fits to a two-exponential expression with equal amplitudes for the fast and slow components for the β- and α-chains, respectively. In the case of Hb-βV[67](E11)T the rates of the fast and slow phases were almost equal and the data could be represented almost as well by a single-exponential expression with $k'_{O_2} = 35\ \mu M^{-1}\ s^{-1}$. Since the β rate constant in native R-state hemoglobin is 100 μM^{-1} s^{-1}, the results in Figure 1 imply a 2–3-fold decrease in $k'_{R\beta}$ for O_2 binding in the mutant protein.

In contrast to the results for O_2 association, the time courses for O_2 replacement by carbon monoxide for the native and mutant proteins were almost indistinguishable, suggesting that the change in polarity at position β[67](E11) has little effect on the rate of O_2 dissociation from fully liganded hemoglobin. Also, the time courses for CO recombination to partially photolyzed samples were virtually identical for the native and mutant tetramers, indicating that k'_{CO} was the same for the native and Thr[67](E11) β-subunits. In contrast, the CO dissociation rate constant, measured by displacement with

Table IV: Rate and Equilibrium Constants for O_2 and CO Binding to the α- and β-Subunits within Oligomeric R-State Native Human Hemoglobin A_0 and Hb-βV^{67}(E11)T[a]

protein	k'_{O_2} ($\times 10^{-6}$ M^{-1} s^{-1})	k_{O_2} (s^{-1})	K_{O_2} ($\times 10^{-6}$ M^{-1})	k'_{CO} ($\times 10^{-6}$ M^{-1} s^{-1})	k_{CO} (s^{-1})	K_{CO} ($\times 10^{-6}$ M^{-1})
HbA_0						
α(native)	28 ± 9 (34)[b]	12 ± 3 (13)	2.3 ± 0.9 (2.6)	2.9 ± 0.5 (2.8)	0.0046 ± 0.0015 (0.0044)	630 ± 240 (640)
β(native)	100 ± 13 (87)	22 ± 8 (34)	4.5 ± 1.7 (2.6)	7.1 ± 2.4 (6.8)	0.0072 ± 0.0028 (0.0084)	990 ± 510 (840)
Hb-βV^{67}(E11)T[b,c]						
α(native)	22 ± 6	7.3 ± 2	3.0 ± 1.2	3.5 ± 0.9	0.0075 ± 0.0020	470 ± 200
β(Thr-E11)	42 ± 10	18 ± 4	2.3 ± 0.9	7.1 ± 2.4	0.0160 ± 0.0040	490 ± 200
pig myoglobin						
native	17 ± 3	14 ± 3	1.2 ± 0.4	0.78 ± 0.16	0.019 ± 0.004	41 ± 13
Thr-E11	2.8	39	0.072	0.61	0.079	7.7

[a] In 0.1 M Bis-tris and 0.1 M KCl, pH 7.0, 20 °C. Association rate constants, k', were obtained from partial photolysis experiments and the dissociation rate constants, k, from replacement reactions as described by Mathews et al. (1989). The association equilibrium constants were calculated from the ratio of the kinetic parameters. All the kinetic traces were fitted to a two-exponential expression with equal amplitudes; the larger rate constants were assigned to β-subunits and the smaller to α-subunits. The parameters for native hemoglobin were taken from Mathews et al. (1989) and those for pig myoglobin from Smerdon et al. (1991). [b] The values in parentheses are the parameters derived for HbA_0 in our control experiments which were carried out at the same time as the experiments with the mutant hemoglobin. [c] The errors for the mutant hemoglobin parameters were computed assuming ±25% for the rate constants and ±40% for the equilibrium constants, based on the data for native hemoglobin taken from Mathews et al. (1989).

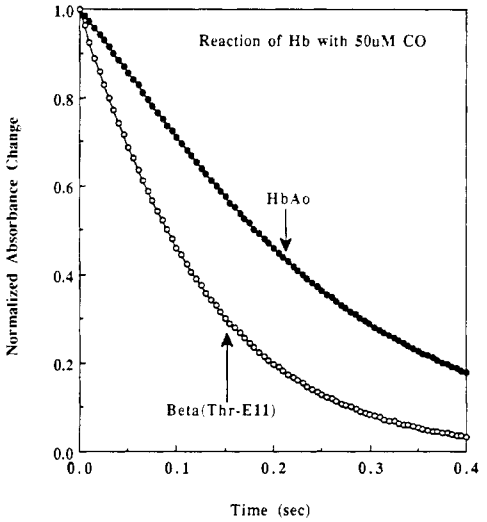

FIGURE 5: Normalized time courses for the reaction of deoxyhemoglobin (5 μM heme) with 50 μM CO at 20 °C in 100 mM Bis-tris and 100 mM KCl at pH 7.0. HbA_0: normal human hemoglobin. β(Thr-E11): mutant hemoglobin in which Val^{67}(E11) was replaced by Thr.

NO, increased 2-fold when Val^{67}(E11) was replaced by Thr (Table IV).

Thus, for both CO and O_2 the Val^{67}(E11) to Thr substitution caused a roughly 2-fold decrease in the equilibrium affinity for the binding of the fourth ligand molecule; however, this effect was expressed differently in the association and dissociation rate constants. These data and the detailed description of their analysis are summarized in Table IV.

Kinetics of CO Binding to Deoxyhemoglobin. Overall time courses for CO binding to native and mutant deoxyhemoglobins are shown in Figure 5. These data indicate that the βVal^{67}-(E11) to Thr substitution causes about a 2–3-fold increase in the rate for the first step in CO binding to β-subunits within deoxyhemoglobin (Table V). This is quite surprising since the βVal^{67}(E11) to threonine substitution caused little change in the association rate constant for the last step in CO binding to R-state β-subunits and a decrease in the corresponding rate constant for O_2 binding.

DISCUSSION

Conformational Data. The similarity, in the Soret region, of the absorption and CD spectra of recombinant wild-type and mutant Hbs, both in the cyanomet and in the deoxy form, suggests that the geometry of the heme pocket was not altered in Hb-βV^{67}(E11)T (Hsu & Woody, 1971). The absence of spectral changes indicates that the mutation did not result in the coordination of a water molecule with iron. Thus, the mutation appears to be conservative since even small changes in the heme coordination complex should result in significant changes in the electronic spectra (Wang & Brinigar, 1979). Changes in the secondary structure were not detected by CD measurements in the far UV, and sedimentation velocity measurements indicated that the hydrodynamic characteristics of Hb-βV^{67}(E11)T were the same of those of HbA_0. These data indicate that the mutation has not altered the conformational characteristics of Hb-βV^{67}(E11)T either in the oxy or deoxy conformation, consistent with the O_2 equilibrium binding data. Smerdon et al. (1991) also observed little change in the tertiary structure of pig Mb when Val^{68}(E11) was replaced with Thr.

Table V: Empirical Analyses of the Time Courses for the Reaction of Excess CO with T-State Natural and Mutant Hemoglobins, and Estimated Association Rate Constants for the First Step of CO Binding[a]

	k' (μM^{-1} s^{-1})	$k'_{T\alpha}$ (μM^{-1} s^{-1})	$k'_{T\beta}$ (μM^{-1} s^{-1})
HbA_0	0.17	0.17	0.17
Hb-βV^{67}(E11)T	0.32	0.17	0.47

[a] The reactant concentrations after mixing were 5 μM heme and 25 μM CO. Buffer: 100 mM Bis-tris and 100 mM KCl at pH 7.0, 20 °C. The binding was followed at 436 nm. Values of $k'_{T\alpha}$ and $k'_{T\beta}$ were calculated assuming that $k'_{obs} = {}^1/_2(k'_{T\alpha} + k'_{T\beta})$ and that $k'_{T\alpha} = k'_{T\beta}$ for native HbA_0. For the mutant, $k'_{T\beta(Thr67)}$ was calculated as $2k'_{obs}$(mutant) $- k'_{T\alpha}$(native). These values are estimated only since we are assuming the overall fitted rate constant applies to the first step in ligand binding to deoxyhemoglobin. The limitations and validity of this assumption are discussed in detail by Mathews et al. (1991).

Kinetic Data. The βVal^{67}(E11) to Thr mutation causes an ~2-fold decrease in the equilibrium association constants for the last step in both CO and O_2 binding to β-subunits (Table IV). This decrease in affinity is expressed differently in the kinetic constants for the two ligands. In the case of O_2 binding, the association rate constant, k'_{O_2}, is decreased ~2-fold and the dissociation rate constant, k_{O_2}, is unchanged. In the case of CO binding, the opposite effect is observed; k'_{CO} is unaffected and k_{CO} increases ~2-fold. When ValE11 is replaced by Thr in pig myoglobin, similar but much larger effects were observed (Table IV). The association rate

constant for O_2 binding decreased 6-fold, and the rate constant for CO dissociation increased 4-fold.

Assignment of the rate parameters obtained from the partial photolysis and replacement reactions to specific Adair intermediates or the R quaternary state is potentially ambiguous. These problems have been described by Mathews et al. (1989). In partial photolysis experiments using the laser system and 1 atm of O_2, the observed rates represent primarily the characteristics of the conformations present in $Hb_4(O_2)_4$ species. Under these conditions, O_2 rebinding is faster than most of the R to T conformational transitions, and the heme concentration is ≥100 μM so that only a small fraction of dimers is present. For the replacement reactions and the CO partial photolysis experiments, low heme concentrations were used (5–10 μM) and the proportion of dimers was 50–80%. Mathews et al. (1989) and Vandergriff et al. (1991) have argued that ligand replacement and the partial photolysis rate constant for O_2 binding are independent of heme concentration for native human hemoglobin at neutral pH in the absence of organic phosphates. Experimentally, liganded dimers appear to exhibit the same O_2 binding parameters as R-state tetramers. Thus, the parameters in Table IV can, at least to a first approximation, be assigned to the R-state in the MWC model. Assignment of these rate constants to the $Hb_4X_3 + X \rightarrow Hb_4X_4$ reaction may be less valid, particularly in the case of the mutant, since the Hb_4X_3 intermediate appears to have a substantial population of T-state-like conformers.

The relatively small effect of the Val^{67}(E11) to Thr substitution in R-state β-subunits supports the previous idea of Mathews et al. (1989) that the distal pocket in β-subunits is intrinsically less polar than that in either α-subunits or myoglobin. Rohlfs et al. (1990), Tame et al. (1991), and others have argued that noncovalently bound water is hydrogen bonded to the distal histidine in deoxy α-subunits and deoxymyoglobin and that this water must be displaced before ligands can approach the heme iron atom. Smerdon et al. (1991) have shown that the β-OH of Thr^{68}(E11) forms a hydrogen bond with the carbonyl oxygen atom of the E7 residue in the mutant pig myoglobin. The nonbonding electrons on the Thr hydroxyl stabilize distal pocket water by additional hydrogen bonding, causing marked decreases in the association rate constants for O_2, NO, and azide binding. Since the rate of CO binding is limited primarily by reaction at the iron atom, k'_{CO} is less affected by distal pocket water and other kinetic barriers which restrict ligand movement through the protein.

In the case of hemoglobin deoxy β-subunits, no water is seen attached to His^{63}(E7), and thus the addition of Thr^{67}(E11) is expected to have less of an effect on ligand binding kinetics. It is possible that βThr^{67}(E11) could form a hydrogen bond with βHis^{63}(E7). However, the effect on the O_2 dissociation rate constant in β-subunits would be expected to be minimal since no hydrogen bond between HisE7 and bound O_2 appears to occur (Phillips, 1981; Mathews et al., 1989). In pig myoglobin there is a strong, favorable interaction between His^{64}(E7) and the second bound O_2 atom, and disruption of this hydrogen bond by Thr^{68}(E11) causes a marked increase in the rate constant for O_2 dissociation (Smerdon et al., 1991).

In all cases, the kinetic measurements show the presence of small modifications, just above the limits of experimental errors (Table IV). The absence of large modifications in the kinetics of ligand binding is also consistent with the thermodynamic analyses of equilibrium O_2 binding, which indicate the absence of substantial changes in oxygen affinity in either the T-state or R-state of the mutant hemoglobin. The 2-fold decrease in $K_{\beta R}$ (Table IV) predicts only a 33% decrease in K_4 in eq 4.

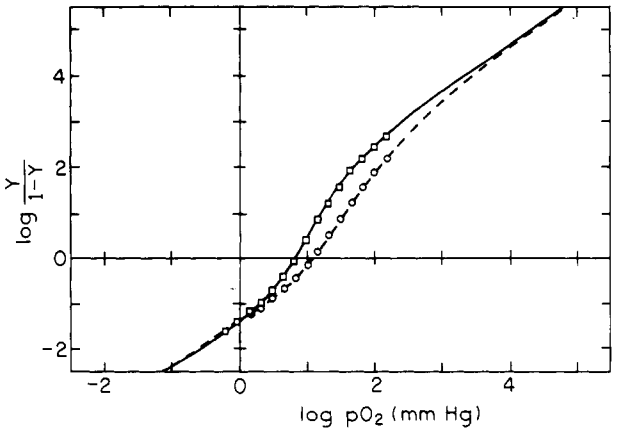

FIGURE 6: Simulated Hill plots of HbA_0 (—) and Hb-βV^{67}(E11)T (- - -) obtained using the intrinsic affinity constants, K_i, listed in Table V. The symbols represent the experimental values.

The 3-fold increase in the rate of CO binding to T-state Thr^{67}β subunits was unexpected. In the native state quaternary structure, the γ_2-CH_3 group of βValE11 and the Nε atom of βHis-E7 are much closer to the iron atom and hinder the rate of ligand binding sterically (Perutz et al., 1970; Mathews et al., 1991). If the hydroxyl of βThr^{67}(E11) caused stabilization of distal pocket water, hindrance to ligand binding would be enhanced, and the CO association rate constant should have decreased instead of increasing. One tentative explanation is that the imidazole side chain of His-E7 rotates about its C_β–C_γ bond to form a direct hydrogen bond with the Thr-E11 hydroxyl. This would partially alleviate the hindrance imposed by the distal histidine, perhaps increasing the rate of CO binding. Verification of this idea will be possible upon high resolution of the diffraction pattern of crystals of Hb-βV^{67}-(E11)T, at present under investigation.

Thermodynamics of Oxygen Binding. The thermodynamic analyses of the oxygen equilibrium curves according to the two-state MWC model indicate that the decreased oxygen affinity of the mutant hemoglobin is due to an increase of the allosteric constant L_0, while the affinities of the initial T- and final R-state are unmodified. The sequential model of Adair was consistent with this picture in indicating that the initial and final oxygenation steps had the same affinity constants in the normal and mutant proteins. In this model the mutation affects the affinity constant of the intermediates, especially for the third step of oxygenation.

However, in this analysis fixing K_4 to the value based on the kinetic results in Table IV is not strictly valid since the Hb_4X_3 intermediate appears to have a greater than 50% population of T-state-like tetramers. In the replacement reactions, there were substantial populations of R-state Hb_2X_2 dimers, and in the O_2 partial photolysis experiments conformational relaxation to T-state structure may not have time to occur. Even with the quantitation problem described above, both analyses are consistent in showing that the decreased affinity of Hb-βV^{67}(E11)T is due to a different distribution of the oxygenated forms with respect to HbA_0. This is illustrated in Figure 6, which presents the simulated Hill plots for HbA_0 and Hb-βV^{67}(E11)T obtained using the affinity constants K_i listed in Table II; the symbols represent the experimental data. The two curves appear to converge to the same asymptotes as expected from the similarity of the K_T and K_R values in the two hemoglobins (Table III); they diverge

in the middle portion, which is defined by the intermediates. This indicates that the effect of the mutation is not expressed by large modifications of the R- and T-states but is confined primarily to the intermediate steps of oxygenation, which in the sequential model are individually analyzed and in the MWC model are included within the allosteric constant L_0. The results suggest that the perturbation introduced by the mutation preferentially stabilizes deoxyhemoglobin-like intermediates during oxygenation. The increased preferential binding of Cl^- and the modified Bohr effect are probably an expression of this modification.

Formation of methemoglobin was not observed in the kinetic experiments, but in the oxygen equilibrium measurements some methemoglobin was formed at the last steps of deoxygenation at very low partial pressure of O_2. The effect of methemoglobin formation on the oxygen equilibrium curve is difficult to assess. The similarity of the T- and R-states in natural and mutant hemoglobin suggests that methemoglobin formation did not produce a distortion of the oxygen equilibrium data. It is very likely that, if the modification observed at the level of the intermediate steps of oxygenation were solely due to methemoglobin formation, this phenomenon would also be reflected in the other parameters. In fact, oxygen equilibrium measurements by Cordone et al. (1990) indicate that constant K_T is the one most affected by the presence of methemoglobin. In oxygen equilibrium measurements, the presence of methemoglobin produces an apparent increase in oxygen affinity (Cordone et al., 1990): It is reassuring that the same 2-fold decrease in oxygen affinity was obtained with both the equilibrium and the kinetic experiments, even though in the latter methemoglobin was not formed.

The relative insensitivity of the β-subunits to heme pocket substitutions seems to be a general phenomenon (Nagai et al., 1987; Mathews et al., 1989, 1991). The only exception is the $Val^{67}E11 \rightarrow$ Ile mutation in which the larger *sec*-butyl sterically hinders access to the β-heme iron atom. Thus the heme pocket, in β-subunits, has a higher plasticity with respect to myoglobin and α-subunits which can compensate for potentially destabilizing amino acids.

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