Hemoglobin Brockton [β138 (H16) Ala → Pro]: An Unstable Variant near the C-Terminus of the β-Subunits with Normal Oxygen-Binding Properties†

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ABSTRACT: Hemoglobin Brockton [β138 (H16) Ala → Pro] is an unstable variant associated with a mild anemia. It has the same electrophoretic mobility as and cannot be resolved from Hb A. Oxygen affinity measurements of blood and hemolysate do not indicate biphasic oxygen saturation, showing that the functional properties of the variant are very similar to those of Hb A. This implies that the introduction of proline into the H-helix at position 138 does not disrupt the critical inter- and intrasubunit hydrogen bonds and salt bridges at the β carboxyl-terminal dipeptide, since these polar interactions are essential for the normal oxygen-binding properties of hemoglobin. X-ray crystallographic data are consistent with these findings and show that the consequences of the β138 Ala → Pro substitution are almost entirely confined to the immediate vicinity of the mutation site. Instability probably results from the inability of a buried hydrogen bond to form between Pro 138β and Val 134β.

The amino acid residues of the carboxyl-terminal region of both the α- and β-chains play critical roles in the oxygen-linked functions of hemoglobin (Perutz, 1970). Abnormal hemoglobins with structural alterations occurring in these regions show markedly altered functional properties, such as changes in oxygen affinity, reduction in cooperativity and Bohr effect, instability, and changes in response to organic cofactors (Bunn & Forget, 1986; Kilmartin & Hewitt, 1971; Perutz & Ten Eyck, 1971). With substitutions involving proline, there are almost always changes in these major functional properties (Wrightstone, 1984) because of the special role that this imino acid plays in the folding of globular proteins (Levitt, 1981). Hemoglobin Brockton β138 (H16) Ala → Pro is unstable but exhibits normal oxygen-binding properties. The latter finding appears inconsistent with the location of the mutation in the carboxyl-terminal region of the β-subunits and the notion that a proline should disrupt α-helical structure. We describe here the properties of this variant and provide structural data to account for its normal functional properties.

Experimental Procedures

Electrophoretic methods for identifying abnormal hemoglobins have been reported (Schneider, 1978). Erythrocytes were examined for inclusion bodies and the heat and 2-propanol procedures used to test for the presence of unstable hemoglobins (Rieder, 1970; Carrell & Kay, 1972). Hemoglobin F was quantitated by alkali denaturation, and Hb A_2 was determined by a microchromatographic method (Singer et al., 1951; Efremov et al., 1974). Isoelectric focusing was carried out on commercial PAG plates pH 5.5–8.5 (LKB) according to the manufacturer's instructions.

Abnormal globin chain for structural analysis was isolated by the Clegg procedure (Clegg et al., 1966) following precipitation of the abnormal hemoglobin with 2-propanol and removal of heme with acid–acetone. The chain was chemically modified with ethylenimine (Raftery & Cole, 1963) and then digested with trypsin (Smyth, 1967). Peptide maps were prepared by chromatography in a solvent of isoamyl alcohol/pyridine/water (7:7:6 v/v), and electrophoresis was done at pH 6.4 for 150 min at 28 V/cm (Bennett, 1967). Peptides resolved by ion-exchange column chromatography and by reverse-phase HPLC (Jones, 1970; Moo-Penn et al., 1983) were hydrolyzed in 6 N HCl at 110 °C for 24 h in vacuo, and the amino acid compositions were determined by using a Beckman 121 amino acid analyzer. Sequence data were obtained from a Beckman 890C sequencer (program 121078) with treatment of the spinning cup with polybrene. The PTH amino acids were identified and quantitated by the method of Somack (1980).

For oxygen equilibrium studies, hemolysates were prepared without organic solvents (Shih et al., 1980). The stroma was removed by precipitation with high salt concentrations followed by ultracentrifugation. Organic phosphates and other ions

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were removed by column chromatography on a mixed-bed resin, and the oxygen equilibrium of the stripped hemoglobin was determined at pH 7.0, 7.4, and 7.9 in 0.1 M phosphate buffer at 25 °C according to the method of Imai (1981). A Cary 219 spectrophotometer and an oxygen electrode (Beckman Instruments No. 39065) were employed as described by Shih (Shih & Jones, 1986). Data acquisition and reduction were done with a PDP 11/V03 computer (Digital Equipment Co.).

For the X-ray studies, the Hb Brockton hemolysate was crystallized in the deoxy state from phosphate-buffered solutions (pH 6.8) of concentrated ammonium sulfate according to the method described by Perutz (1968) for pure deoxyhemoglobin A. Since all the hemoglobin crystallized in these solutions, we assumed that the crystals contained about 30% Hb Brockton and 70% Hb A. Other examples of crystallization and analysis of mixtures of variant hemoglobins and Hb A have been reported (McDonald et al., 1980; Moo-Penn et al., 1984). Single crystals of the deoxyhemoglobin Brockton/deoxyhemoglobin A mixtures were mounted in quartz capillary tubes in an atmosphere of pure nitrogen. The diffraction data were collected to a resolution of 3.0 Å with an Enraf-Nonius CAD4 diffractometer using the Ω-scan mode. Two crystals were used to collect the data so that degradation due to radiation damage never exceeded 10% as determined by repeated measurements of four standard reflections. An empirical correction for radiation damage was applied as described previously (Ten Eyck & Arnone, 1976). The method of North et al. (1968) was used to correct for X-ray absorption. The difference electron density map was produced by using the difference amplitudes ($|F|$ Hb Brockton/Hb A − $|F|$ Hb A) together with phases calculated from the refined coordinates of deoxyhemoglobin A (Arnone et al., 1986). Since the difference map displayed a high level of 2-fold symmetry, it was averaged about the noncrystallographic dyad that relates the two equivalent $\alpha\beta$ dimers.

The structural data used for model-building analysis were obtained from the Brookhaven Protein Data Bank via the PROPHET computer network. The coordinates for adenylate kinase were reported by Schultz et al. (1974) and those for glyceraldehyde-3-phosphate dehydrogenase by Moras et al. (1975). Model helices containing an internal proline residue were superimposed onto the H-helix of the deoxyhemoglobin β-chain by using the MATCHMOL procedure of the PROPHET system or the least-squares procedure of Kabsch (1976).

Results and Discussion

The proposita was a 9-year-old Caucasian female who presented with a mild anemia when she was first studied in 1978. Her hemoglobin concentration was 10.5 g/dL, hematocrit 34%, and MCHC 30.88%. The red cell 2,3-diphosphoglycerate concentration was 6.94 μmol/mL RBC or 22.5 μmol/g hemoglobin. An evaluation of her hemolysate by electrophoresis at pH 8.5 on cellulose acetate and on citrate agar at pH 6.3 revealed patterns identical with those of Hb A. Similar results were also obtained for globin chain analyses at pH 6.0 and pH 8.9 in urea–mercaptoethanol buffers. In addition, isoelectric focusing did not reveal the presence of an abnormal hemoglobin. However, the heat and 2-propanol tests for unstable hemoglobin were positive, and inclusion bodies were observed when erythrocytes were treated with brilliant cresyl blue. The concentration of the unstable component was estimated to be 30% by heat denaturation in 0.02 M phosphate buffer, pH 7.0 at 60 °C. The concentration of Hb A_2 was 3.4%, and the alkali-resistant hemoglobin was 1.6%. The abnormal hemoglobin was not found in her parents, which indicates that Hb Brockton is a spontaneous mutation.

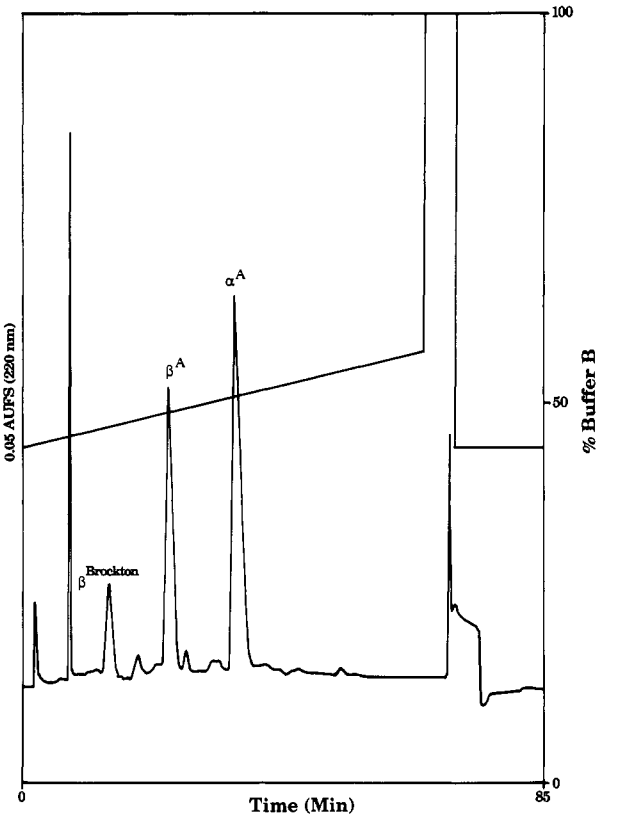

FIGURE 1: Separation of globin chains by HPLC on a Vydac C4 column (4.6 mm × 25 cm, The Separation Group). Approximately 50 μg of hemoglobin was applied. Buffer A was 80 mL of a 0.1% TFA/water solution made up to 100 mL with a solution of 0.1% TFA in acetonitrile. Buffer B was 40 mL of 0.1% TFA/water and 60 mL of 0.1% TFA in acetonitrile. The linear gradient was 44% buffer B at time zero to 56.5% buffer B at 65 min. The flow was 1 mL/min at room temperature.

Table I: Hemoglobin Brockton Amino Acid Composition of βT-14,15[a]

amino acid	expected	found	amino acid	expected	found
Lys	1	0.9	Ala	4	2.8
His	2	1.7	Val	3	1.5
Asp	1	0.9	Leu	1	1.1
Pro	*0*	*1.0*	Tyr	1	0.7
Gly	1	0.9			

[a] The results are expressed as molar ratios. The number of residues of Val recovered was low because of the Val–Val sequence (133–134) at the N-terminal of the peptide.

From the 2-propanol-precipitated fraction, an abnormal chain was isolated, which eluted from the Clegg column in the position of βA-chain. There was a small fraction of αA-chain. Later, all three chains could be separated directly from the hemolysate by reverse-phase HPLC (Figure 1). The percentage of β-Brockton chain is about 23% of total β-chain in contrast to the 30% variant found by heat denaturation. The difference was attributed to the instability of the variant and the age of the sample on which the HPLC analysis was done.

A peptide map of a tryptic digest of the abnormal chain revealed a normal distribution of peptides, except that βT-14 was shifted, appeared to be more hydrophilic, and migrated more rapidly to the cathode. Column chromatography on the cationic resin Aminex-A5 (Bio-Rad) also shows that the defect was in βT-14. This peptide that normally elutes with βT-4 instead elutes later in a cluster of peaks comprising βT-1, βT-10, and βT-11 (Johnson et al., 1980). Rechromatography of this cluster of peptides on Dowex 50-X2 resulted in the

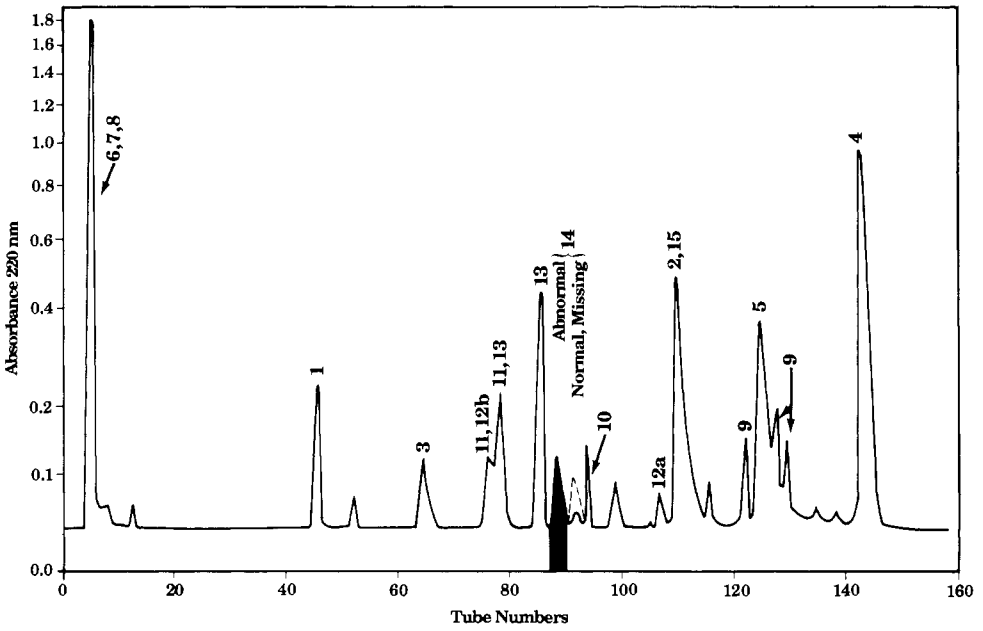

FIGURE 2: Chromatogram of tryptic peptides resolved on a Waters μBondapak C18 column. Buffer A was 10 mM sodium acetate, pH 5.68, and buffer B was a 1:2 dilution of buffer A with acetonitrile. The gradient was 1–60% buffer B in 120 min at room temperature. The flow was 1.5 mL/min, and 0.5-mL fractions were collected.

Table II: Sequence of βT-14 from Hemoglobin Brockton[a]

amino acid	nmol	amino acid	nmol
Val	23.0	*Pro*	8.5
Val	15.0	Asn	2.6
Ala	11.4	Ala	11.4
Gly	7.4	Leu	2.9
Val	20.7	Ala	4.1

[a] The experimental details are given in the text. The initial sample was 45 nmol of peptide purified by HPLC.

isolation of abnormal βT-14,15. The amino acid composition of this peptide (Table I) shows clearly that the substitution is Ala → Pro. In subsequent studies, it was more convenient to prepare βT-14 by HPLC (Figure 2). Under these conditions, βT-14 was shifted slightly upstream from the normal. Of the four residues of alanine in βT-14, sequence analysis showed the residue at 138 to be proline (Table II).

The oxygen affinity properties of Hb Brockton together with Hb A are summarized in Table III. A red cell suspension of the patient's blood produced a slight shift of the equilibrium curve to the right, giving a P_{50} of 29 mmHg compared with a P_{50} of 26.5 mmHg for a normal control. The shape of the curve was completely sigmoid, and this slight shift can be explained by the relatively high levels of 2,3-diphosphoglycerate.

Studies of hemolysates that were deionized by passage through a column of mixed-bed resins indicate that the equilibrium curves of the "stripped" hemoglobins were not significantly different from those of normal controls. The P_{50} and n values and the shape of the curves at pH 7.0, 7.4, and 7.9 were all within normal limits (Figure 3). Over the period required to complete all oxygen equilibrium measurements, the optical density of the hemolysate solution at 560 nm re-

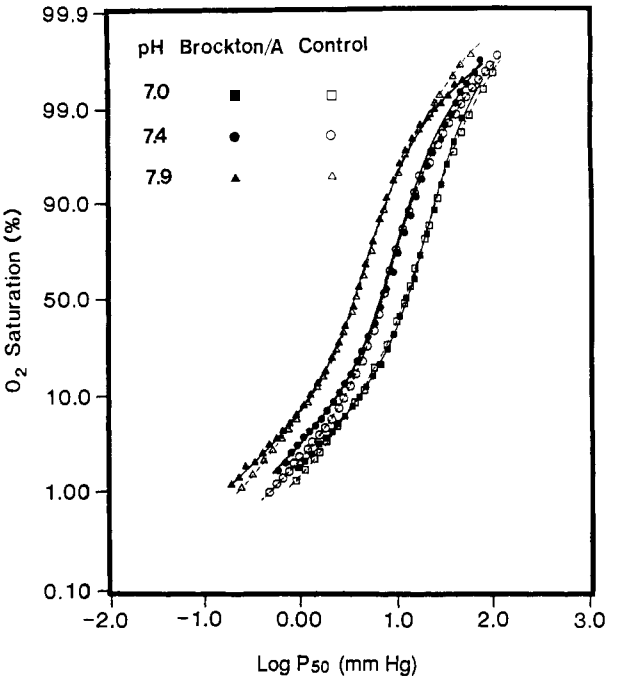

FIGURE 3: Oxygen saturation curves of Hb Brockton/Hb A hemolysates in 0.1 M potassium phosphate buffer at 25 °C at various pHs. The hemoglobin concentration was 60 μM (heme).

Table III: Oxygen Affinity Properties of Hemoglobin Brockton

exptl conditions	Hb Brockton/ Hb A P_{50}	Hb Brockton/ Hb A n	normal Hb A P_{50}	normal Hb A n	normal range P_{50}	normal range n
red cell suspension–0.15 M potassium phosphate buffer, pH 7.4, 37 °C	29.0	3.0	26.5	3.1	26–28	2.9–3.2
hemolysate (60 μM heme)–0.10 M potassium phosphate buffer, 25 °C						
pH 7.0	13.8	2.7	12.6	2.8	12–14	2.7–3.1
pH 7.4	7.8	2.8	7.8	2.8	7.5–8.5	2.7–3.2
pH 7.9	4.1	2.7	4.0	2.8	3.8–4.4	2.6–3.1

[a] The P_{50} values are expressed as mmHg; n values represent the cooperativity of the hemoglobin oxygen binding obtained from Hill plots.

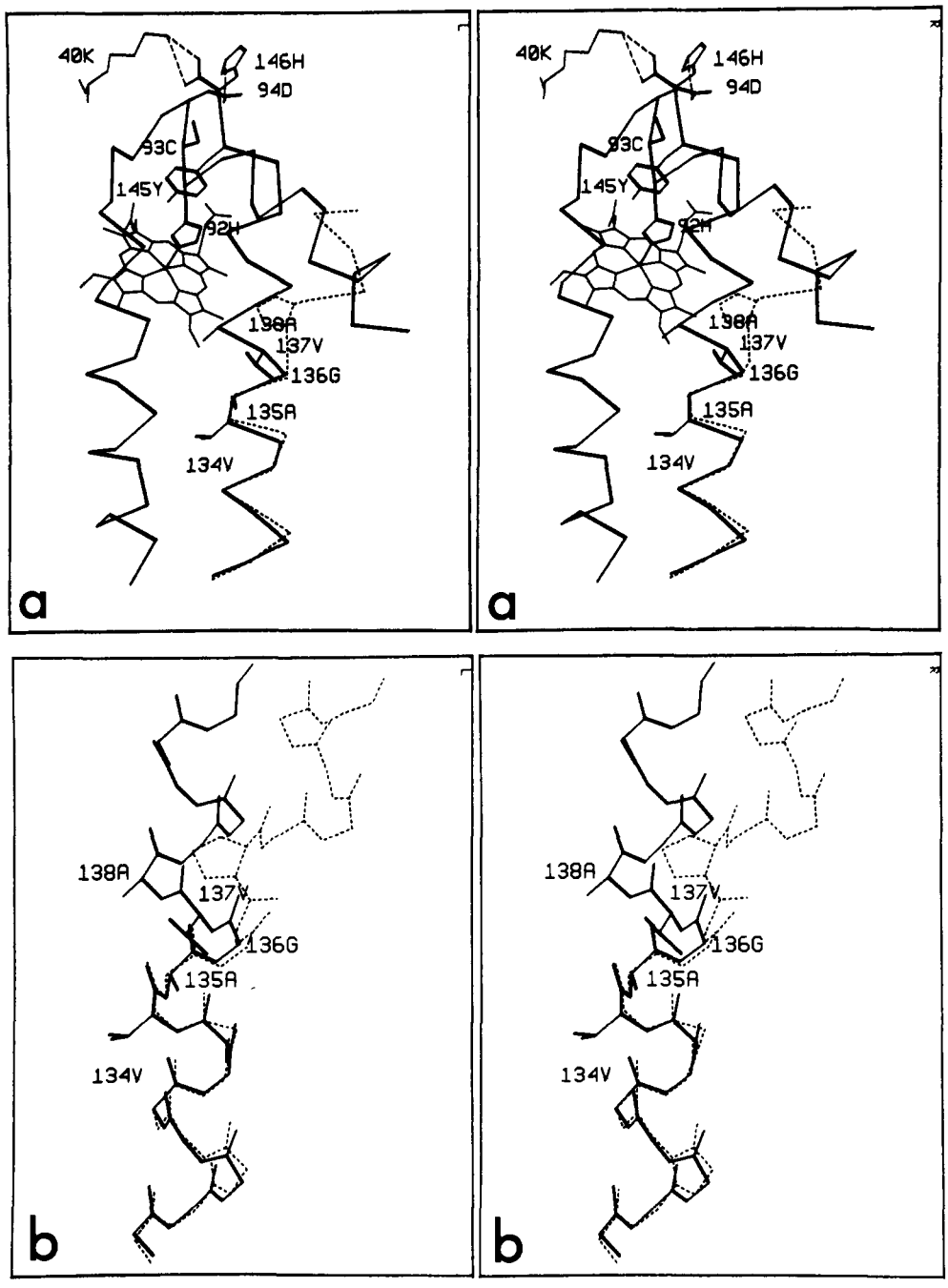

FIGURE 4: Stereo drawings of the mutation site (residue 138β) in deoxyhemoglobin A with the adenylate kinase 144–164 helix (dotted lines) superimposed on the β-subunit H-helix (see text). (a) α-Carbon plot of β-subunit residues 127–146 and 80–115. The β-heme is shown along with the side chains of selected β-subunit residues and the side chain of Lys 40α. Also shown is the His 146β–Asp 94β ionic contact and the His 146β–Lys 40α ionic contact across the $\alpha_1\beta_2$ interface. The α-carbons of adenylate kinase residues 148–164 and the side-chain atoms of Pro 159 are linked with dotted lines. (b) Enlarged view of the mutation site showing the backbone atoms and selected side chains of the superimposed β-subunit and adenylate kinase helices.

mained approximately the same, indicating that there was no significant degradation of Hb Brockton (due to instability) during the analyses. These results suggest that the oxygen-binding properties of the variant must be very similar to those of normal Hb A.

There was some concern regarding the interpretation of the data since the oxygen-binding studies were based on a mixture of approximately 30% Hb Brockton and 70% Hb A. Initially, a review of similar mutations and the known tendency of proline to distort α-helical structures led us to expect that the 138β Ala → Pro substitution should have caused major functional changes because of disruptions in the critical C-terminal area of the β-chain. Indeed, if this were the case, Hb Brockton in whole cells and in hemolysate should have produced a biphasic, left-shifted oxygen dissociation curve. Such findings have been reported for many variants with concentrations similar to those of Hb Brockton (Marti et al., 1976; Herano et al., 1981; Jensen et al., 1975; Adamson et al., 1969; Wajcman et al., 1975). Because of our inability to separate the variant from Hb A, the data do not provide an unequivocal answer to the oxygen-binding properties of Hb Brockton. However, the data show that if any functional differences exist between Hb Brockton and Hb A, these must be relatively minor.

Before collecting X-ray data on deoxyhemoglobin Brockton, we constructed models of the altered H-helix to judge the potential structural perturbation caused by the mutation. The models were based on the known 3-dimensional structures of

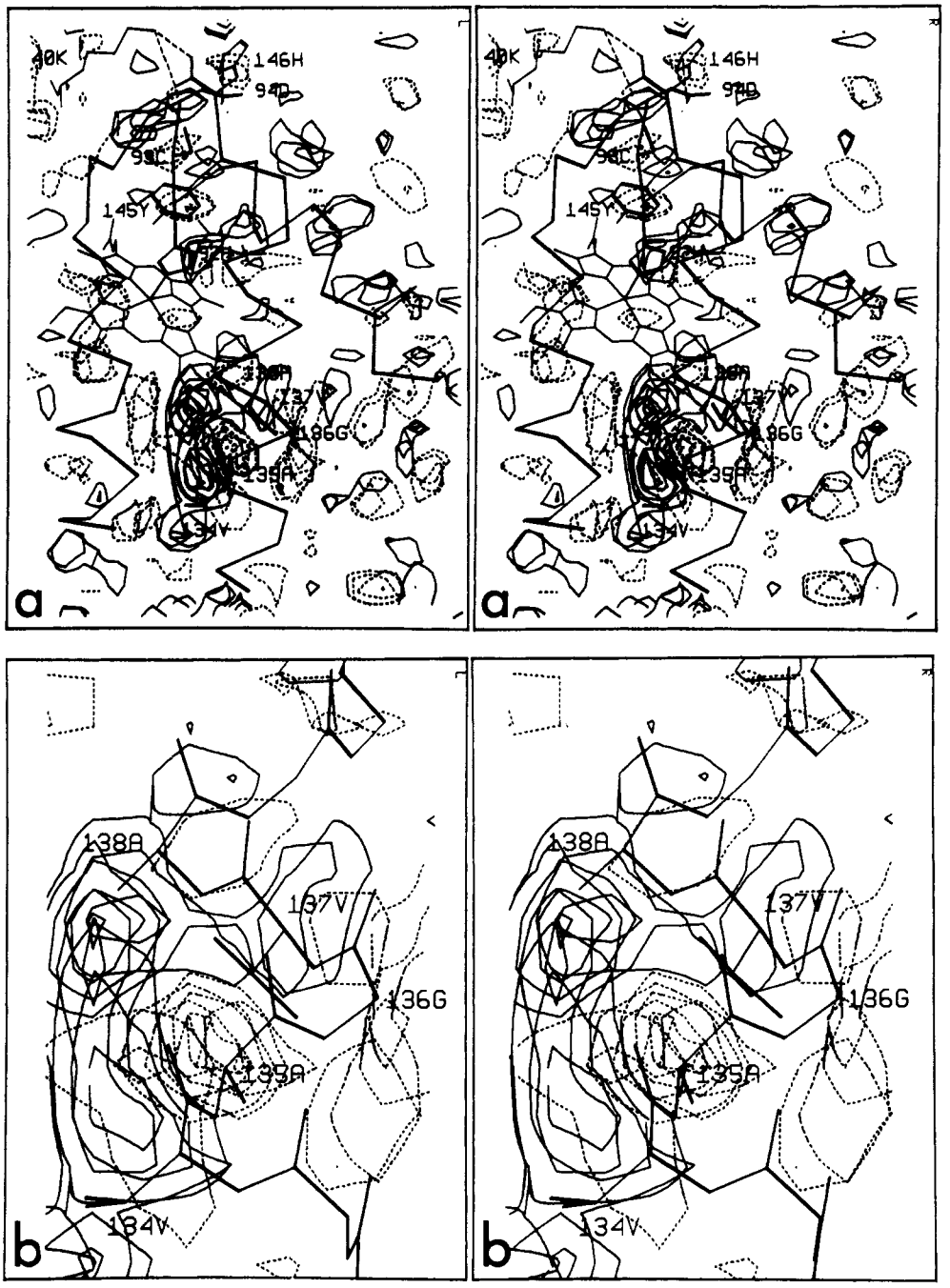

FIGURE 5: [Deoxyhemoglobin Brockton – native deoxyhemoglobin] difference electron density map superimposed on the atomic model of human deoxyhemoglobin. The map has been contoured in intervals of 1.5σ, where σ is defined as the root mean square density of the entire map. Positive difference density features are shown as solid contours, negative difference density as dashed contours. (a) Five contiguous sections (spaced 1 Å apart) of the difference electron density map show that structural changes are limited to the 138 Ala → Pro mutation site. (b) Four contiguous sections (spaced 1 Å apart) of difference electron density show the small structural changes at the mutation site.

helices with internal prolines. In particular, glyceraldehyde-3-phosphate dehydrogenase (Schultz et al., 1974) contains a helix consisting of residues 148–166 that include a proline at position 156, and in adenylate kinase (Moras et al., 1975) residues 144–164 form a helix with a proline at position 159. In both cases, the proline causes a sharp bend of about 20° in the helix axis. The adenylate kinase 144–164 helix is a particularly good match to the globin H-helix (residues 123–143) in that both helices are the same length and have a proline residue in the same relative position. When the backbone atoms of these two helices are superimposed by least-squares fit, the rms deviation is 1.7 Å. However, almost all the deviation occurs at the carboxyl-terminal end of the helices. If only residues 125–135 of the β-chain and residues 146–156 of the adenylate kinase are superimposed, the rms deviation of the backbone atoms is 0.42 Å.

With the two helices superimposed in this way, the potential effect of the "proline kink" on the position of the carboxyl-terminal end of the H-helix is very large (Figure 4). If the H-helix were perturbed to this degree in deoxyhemoglobin Brockton, critical contacts involving Tyr 145β and His 146β would be broken. Specifically, disrupting the intrasubunit hydrogen bond between Tyr 145β and Val 98β, the intrasubunit salt bridge between 146β and Asp 94β, and the intersubunit salt bridge between His 146β_2 and Lys 40α_1 results in complete loss of cooperativity, loss of half the Bohr effect, and about a 20-fold increase in oxygen affinity (Bunn & Forget, 1986; Kilmartin & Hewitt, 1971; Arnone et al., 1976, 1981). Such changes would be evident in the shape and position of the oxygen equilibrium curve of a hemolysate with

the Brockton variant comprising about 30% of the total hemoglobin. The functional data described above lead to the prediction that the β138 proline mutation in Hb Brockton does not cause major changes in the tertiary structure of the chain.

The [deoxyhemoglobin Brockton − deoxyhemoglobin A] difference electron density map is presented in Figure 5. Although this map is somewhat noisy because of the level of the variant hemoglobin (30%), it is clear (Figure 5a) that significant difference electron density features occur only in the immediate vicinity of the mutation site. Consistent with the functional data, there is no evidence for significant structural changes at the β-chain carboxyl-terminal dipeptide. An enlarged view of the difference map in the region of residue 138β (Figure 5b) shows two main features. The first is a positive electron density peak next to the methyl group of Ala 138 that is clearly due to the additional carbon atoms that result from the Ala → Pro substitution. The second feature is a positive/negative pair of difference peaks that straddles the carbonyl oxygen of Val 134β, showing that it shifts away from the axis of the H-helix. This oxygen atom normally forms a strong intrahelix hydrogen bond with the backbone N–H group of Ala 138β. With a proline residue at position 138β, a hydrogen bond is no longer possible, and the carbonyl oxygen at Val 134β shifts to a new position of equilibrium.

The sharp kink in the adenylate kinase and the glyceraldehyde-3-phosphate dehydrogenase helices exposes the polar backbone atoms in the region of the kink to the solvent so that they can form hydrogen bonds with water. In Hb Brockton, a sharp kink in the H-helix at residue 138β cannot occur because it would result in steric collisions with the F-helix and in the exposure of several buried nonpolar residues. Thus, the carbonyl oxygen of Val 134β is buried without hydrogen bonding to a complementary polar group. The complete loss of the Val 134β–Ala 138β hydrogen bond could destabilize the tertiary structure of each subunit (in both the oxy and deoxy quaternary structures) by 2–4 kcal/mol. The movement of the carbonyl oxygen at Val 134β toward the surface of the β-subunit may compensate to some degree for the loss of the H-helix hydrogen bond. However, this oxygen atom will still not be fully accessible to the solvent, and this is probably the main structural reason for the instability of Hb Brockton.

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