Specific Modification of the α Chain C-Terminal Carboxyl Group of Hemoglobin by Trypsin-Catalyzed Hydrazinolysis†

J. V. Kilmartin,* A. Arnone,* and J. Fogg

ABSTRACT: In human deoxyhemoglobin a salt bridge links the α carboxyl of Arg-141 of each α chain to the ϵ-amino group of Lys-127 of the opposite α chain. These salt bridges are believed to contribute to the constraints in the quaternary deoxy (T) structure that lower its oxygen affinity. We have tested this hypothesis by incubating hemoglobin with 2 M hydrazine and trypsin which catalyzes specifically the reversible hydrazinolysis of the α carboxyl of Arg-141α. X-ray analysis shows the major structural difference between native deoxyhemoglobin and hydrazide deoxyhemoglobin to be the loss of the Arg-141α_1–Lys-127α_2 salt bridge and its Arg-141α_2–Lys-127α_1 counterpart. Accurate oxygen equilibrium curves of hydrazide hemoglobin show that blocking of the salt bridge has raised the oxygen affinity of the T structure while leaving that of the quaternary oxy (R) structure unchanged.

The cooperativity of the $\alpha_2\beta_2$ hemoglobin tetramer depends on a reversible transition between two alternative quaternary structures, the low affinity, deoxy (or T) form, and the high affinity, oxy (or R) form. The subunits of the R structure have ligand affinities similar to those of free α and β chains (Noble, 1969). According to the allosteric theory of Monod et al. (1965), the low affinities of the T structure subunits should be caused by constraints placed upon the subunits by a greater number and/or energy of bonds between them. These bonds would be responsible for the free energy of cooperativity in hemoglobin. Perutz (1970) has proposed that these constraining bonds include the salt bridges found in the deoxy- but not the oxyhemoglobin structure. This proposal may be tested by specific chemical modification of a salt bridge followed by measurement of the oxygen affinity of the T structure (K_T).[1] Such a test requires accurate determination of oxygen equilibrium of the modified hemoglobin, for example, by the technique of Imai et al. (1970). Since dissociation into dimers may falsify the results, equilibria must be determined at high protein concentration (Ackers et al., 1975). Unambiguous values of K_T can be calculated from the equilibria provided the modified hemoglobin exhibits reasonable cooperativity ($n \geq 2$) (Baldwin, 1975).

The α carboxyl of Arg-141α_1 forms a salt bridge with the ϵ-amino group of Lys-127α_2 which also interacts with the carboxyl group of Asp-6α_2 (Fermi, 1975). At 3.5-Å resolution, the electron density map of human deoxyhemoglobin also indicated the possibility of an additional salt bridge between the α carboxyl of Arg-141α_1 and the α-amino group of Val-1α_2 (Muirhead and Greer, 1970). However, when the resolution of this map was extended to 2.5 Å (Ten Eyck and Arnone, 1976), refinement of the atomic model by Fermi (1975) showed these groups to be too far apart (5.3 Å) to interact strongly. Instead, recent x-ray studies indicate that the α-amino group of Val-1α_2 forms a salt bridge with an inorganic anion positioned between it and the guanidinium group of Arg-141α_1 (Arnone et al., 1976, 1977; Arnone and Williams, 1977). It is likely that this interaction of the α-amino group of Val-1α with chloride ions makes it responsible for about 20% of the alkaline Bohr effect in 0.1 M Cl^- (Kilmartin et al., 1973; Rollema et al., 1975). All these interactions are disrupted in the R structure (Heidner et al., 1976).

In this paper we have investigated the effect of removal of the salt bridge between the α carboxyl of Arg-141α_1 and the ϵ amino of Lys-127α_2 on the structure and function of hemoglobin by specific modification of the α-carboxyl group by hydrazinolysis. Trypsin can catalyze the removal or addition of a hydrazide from the α carboxyl of a lysine or arginine residue in the following reaction:

$$-CONHNH_2 + H_2O \rightleftharpoons -COOH + NH_2NH_2 \quad (1)$$

The equilibrium constant of this reaction is such that in high concentrations of hydrazine the hydrolysis is reversed and trypsin will catalyze the formation of the hydrazide (Fersht and Requena, 1971; Fastrez and Fersht, 1973). This paper shows that incubation of hemoglobin with trypsin and 2 M hydrazine causes the specific formation of a hydrazide on the α carboxyl of Arg-141α. In the absence of trypsin no reaction occurs.

A difference electron density map of the deoxy form of hydrazide hemoglobin vs. native deoxyhemoglobin clearly shows that the salt bridge between Arg-141α_1 and Lys-127α_2 is broken, with the ϵ-amino group of Lys-127α_2 moving closer to Asp-6α_2. This major structural change causes weaker secondary perturbations which include the loosening of a salt bridge between the guanidinium ion of Arg-141α_1 and Asp-126α_2 and a small shift in the position of Tyr-140α. In addition, the removal of the negatively charged α carboxyl together with a decrease in the distance between Val-1α_2 and Arg-141α_1 causes increased occupancy of the inorganic anion binding site mentioned above. Aside from small movements of atoms adjacent to the reaction site, no other significant structural changes are evident.

Accurately determined oxygen equilibrium curves of hydrazide hemoglobin show a change in K_T, indicating that the salt bridge between Lys-127α_2 and the α carboxyl of Arg-141α_1 contributes to the low affinity of the normal T structure. When the reaction was reversed (eq 1) by digestion of hydra-

† From the MRC Laboratory of Molecular Biology, Cambridge, England (J.V.K. and J.F.), and the Department of Biochemistry, University of Iowa, Iowa City, Iowa (A.A.) *Received May 9, 1977.* The crystallographic research reported here was supported by National Institutes of Health Grant AM-17563, National Science Foundation Grant GB-43803, and a National Institutes of Health Research Career Development Award to A.A.

[1] Abbreviations used: K_1 and K_4, first and fourth Adair constants; P_{50}, oxygen pressure at half saturation; K_T and K_R, association constants (in mm Hg^{-1}) of the T and R states for oxygen; CM, carboxymethyl; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol.

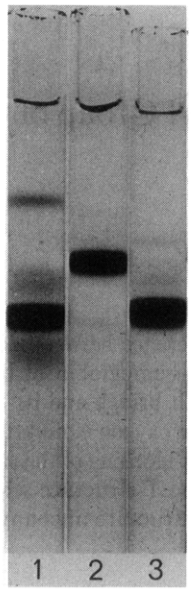

FIGURE 1: Isoelectric focusing between pH 6.0 and 8.0. Tube 1, normal hemoglobin; tube 2, hydrazide hemoglobin; tube 3, hydrazide hemoglobin treated with trypsin.

zide hemoglobin in the absence of hydrazine, the functional properties of the digested hemoglobin reverted to normal (Kilmartin et al., 1975b).

Experimental Procedures

Preparation of Hydrazide Hemoglobin. Human hemoglobin was prepared as described previously (Kilmartin and Rossi-Bernardi, 1971). Hydrazine (4 M), pH 6.5, was prepared by neutralization in ice of hydrazine hydrate (BDH Reagent Grade) with concentrated HCl; 100 mL of this was added to 100 mL of 20 mg/mL CO-hemoglobin (freshly bubbled with CO) containing 2 mg/mL diphenylcarbamoyl chloride treated trypsin (Erlanger and Cohen, 1963) and 5 mM $CaCl_2$. The solution was bubbled with CO again and left 48 h at 4 °C. After 1.6 mg/mL of soybean trypsin inhibitor was added to inhibit the trypsin, the hydrazine was removed by gel filtration against 0.01 M sodium phosphate, pH 6.5 (0.77 g/L $NaH_2PO_4 \cdot 2H_2O$, 0.36 g/L Na_2HPO_4). The reacted hemoglobin was applied to a 6 × 10 cm CM-cellulose (CM-52, Whatman) column equilibrated with the same buffer and eluted with a linear gradient of 10 L of 0.01 M to 10 L of 0.1 M sodium phosphate buffer (pH 6.5). Only two main peaks were eluted, the first was normal hemoglobin and discarded, the second contained about 70% hydrazide hemoglobin and was further purified by preparative isoelectric focusing in polyacrylamide gel between pH 6.0 and 8.0 (Righetti and Drysdale, 1971) to give pure hydrazide hemoglobin.

Reversal of Hydrazide Reaction with Trypsin. The hydrazide was removed from hydrazide hemoglobin by incubation with trypsin as described above except that the 2 M hydrazine was not present. The resulting hemoglobin was further purified by preparative isoelectric focusing.

The amount of hydrazide incorporated into the hemoglobin was measured by incubation of hydrazide hemoglobin, adjusted to pH 6.5, with trypsin (1:10) at 25 °C. At 10-min intervals samples were removed and assayed for hydrazine (Bramholtz et al., 1959); 0.1 mL of hemoglobin solution was added to 3 mL of a solution containing equal volumes of 1.6 N HCl and 4% (w/v) dimethylaminobenzaldehyde in 95% ethanol. The method was calibrated using hemoglobin solutions containing known amounts of hydrazine.

Crystallization and X-Ray Analysis of Hydrazide Hemoglobin. Attempts to crystallize pure hydrazide hemoglobin resulted in crystals which were too small to be used for data collection. However, a mixture of half hydrazide hemoglobin and half hemoglobin A did form large crystals from phosphate-buffered solutions (pH 6.5) of concentrated ammonium sulfate according to the method described by Perutz (1968) for human deoxyhemoglobin A.

Single crystals of native or hydrazide deoxyhemoglobin were mounted in quartz capillary tubes in an atmosphere of high purity nitrogen. The diffraction data were then collected to a resolution of 3.5 Å on an Enraf-Nonius CAD4 diffractometer using the ω scan mode. Two crystals were used for each set of data. Degradation due to radiation damage never exceeded 8% as determined by repeated measurements of four standard reflections. An empirical correction for radiation damage was incorporated into the crystal scaling calculations as described previously (Ten Eyck and Arnone, 1976). We corrected for absorption by the method of North et al. (1968).

Difference electron density maps were calculated using the known phases of deoxyhemoglobin A (Ten Eyck and Arnone, 1976) and the difference amplitudes ($|F|_{\text{hydrazide Hb}} - |F|_{\text{Hb A}}$), and then symmetry averaged about the molecular twofold rotation axis that relates the two equivalent $\alpha\beta$ dimers (Muirhead et al., 1967).

Enzymatic Digestions. Carboxypeptidase A and B digests (Kilmartin et al., 1975a) were carried out for 1 h at 25 °C using enzyme to substrate ratios of between 1:5 and 1:100. Amino acids from peptide maps (Kilmartin and Clegg, 1967) were identified by electrophoresis at pH 2.1 in acetic acid–formic acid–water 8:2:28 (v/v) using standard amino acids as markers.

Measurement of Oxygen Equilibrium Curves. Oxygen equilibrium curves were measured by the method of Imai et al. (1970) using a Gilford 2400 spectrophotometer at 700 nm. A Solatron data logger and digital voltmeter gave simultaneous readings of pO_2 and optical density which were recorded on paper tape and a Hill plot was computed. The protein concentrations used were 20 mg/mL, 10 mg/mL, and 5 mg/mL in 0.2 M Bistris, 0.1 M Cl, pH 6.8, at 25 °C. The wavelength used was 700 nm. The methemoglobin reductase system (Hayashi et al., 1973) was used to prevent excessive methemoglobin formation during measurement of the curve. The components were obtained from Sigma except ferredoxin NADP reductase which was purified from spinach (Forti, 1971); units of enzyme were measured by the ferricyanide assay. The concentrations of the components used were: catalase (type C-100), 1.2 mg/mL; glucose 6-phosphate, 20 mM; glucose-6-phosphate dehydrogenase (type XV), 0.004 mg/mL; NADP, 1.3 mM; ferredoxin (type III), 0.027 mg/mL; and ferredoxin-$NADP^+$ reductase, 1 unit/mL. Methemoglobin before and after the equilibrium curve was 3.3% and 3.8% for hydrazide hemoglobin and 1.5% and 2.5% for normal hemoglobin. K_1 values were measured by hand from the equilibrium curves.

Results

Chemical Characterization of Hydrazide Hemoglobin. Hydrazide hemoglobin was characterized after purification on CM-cellulose and preparative isoelectric focusing. Digestion with carboxypeptidase B (Antonini et al., 1961; Kilmartin et al., 1975a) under conditions where Arg-141α is completely removed from normal hemoglobin released only 0.02 mol of Arg-141α per $\alpha\beta$ dimer, whereas carboxypeptidase A completely released both His-146β (1.0 mol per $\alpha\beta$ dimer) and Tyr-145β (1.2 mol per $\alpha\beta$ dimer). When the hydrazide was

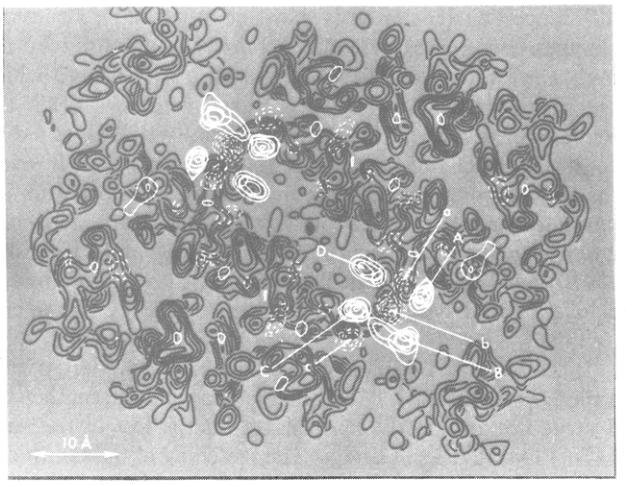

FIGURE 2: The 3.5-Å difference electron density map of hydrazide deoxyhemoglobin (white contours) superimposed on the 2.5-Å native deoxyhemoglobin electron density map (black contours). The native electron density map is a composite of six contiguous two-dimensional sections (sections $y = \pm 10$ to ± 15, space 1 Å apart), contoured at intervals of 0.15 C Å^{-3} above the zero level, with the upper contours obscuring the lower ones. The difference electron density map consists of three sections ($y = +13$ to $+15$, spaced 1 Å apart), contoured at intervals of $\pm$ 0.013 C Å^{-3} with the first contours starting at $\pm$ 0.026 C Å^{-3}. Positive difference electron density is marked by solid white contours and upper case letters. Negative difference electron density is marked by dashed white contours and lower case letters. Both the native and difference electron density maps have been averaged about the molecular twofold axis of symmetry. This axis is perpendicular to the plane of the paper and passes through the center of the figure.

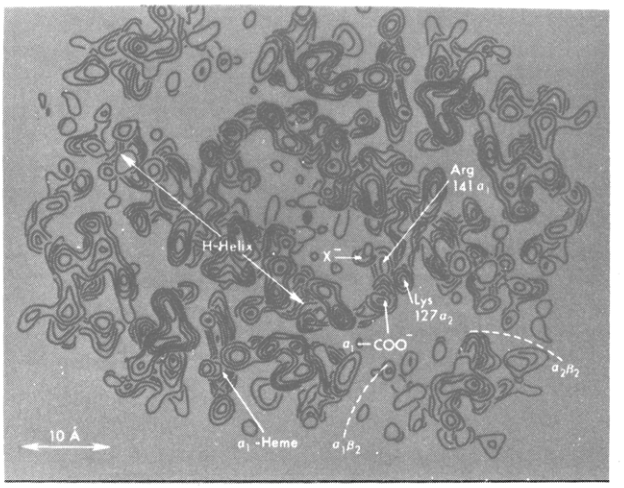

FIGURE 3: Native deoxyhemoglobin electron density as in Figure 2, but with labels to indicate the location of the α_1 chain H-helix, residues Arg-141α_1 and Lys-127α_2, the α_1 chain COOH-terminal carboxyl group, a small portion of the $\alpha_1\beta_2$ and $\alpha_2\beta_2$ interfaces, and the α_1 heme group. X^- is an inorganic anion bound between the α-amino group of Val-1α_2 (not shown) and the quanidinium ion of Arg-141α_2. The counterparts to these features (e.g., Arg-141α_2 and the $\alpha_2\beta_1$ interface) have not been labeled, but are related by the twofold symmetry axis.

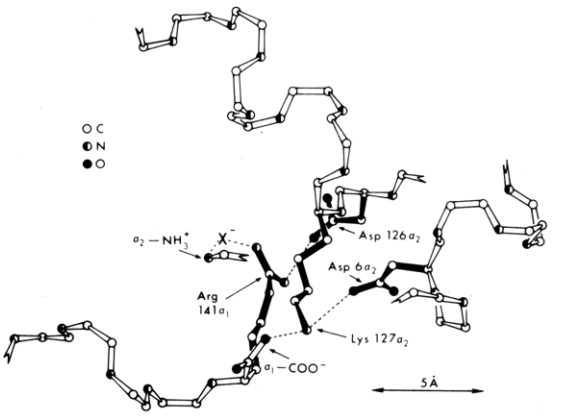

FIGURE 4: Sketch showing the environment of Arg-141α_1 in human deoxyhemoglobin. The COOH terminus of the α_1 chain is labeled α_1-COO$^-$, and the NH_2 terminus of the α_2 chain is labeled α_2-NH_3^+. For clarity, the peptide backbone between the first and fifth residues of the α_2 chain has been deleted.

removed from Arg-141α by incubation with trypsin, up to 0.9 mol of NH_2NH_2 per $\alpha\beta$ dimer was released; the isoelectric point (Figure 1) and oxygen equilibrium curve (Kilmartin et al., 1975b) reverted to normal and Arg-141α was now completely released by carboxypeptidase B digestion (1.0 mol per $\alpha\beta$ dimer). Treatment of des-(Arg-141α)hemoglobin with trypsin and 2 M hydrazine gave no changes in isoelectric point as shown by isoelectric focusing, as did treatment of normal hemoglobin in the absence of hydrazine. Comparison of peptide maps of chymotryptic digests of normal and hydrazide hemoglobin showed one extra ninhydrin positive spot present in normal hemoglobin. This was identified as free arginine by electrophoresis at pH 2.1, showing that chymotrypsin had cleaved between Tyr-140α and Arg-141α. When the pH 6.5 electrophoresis strip of both digests was stained for hydrazides using silver nitrate (Dawson et al., 1969), an extra spot more basic than free arginine was found in hydrazide hemoglobin; normal hemoglobin had no hydrazide positive spots. The extra spot had the same mobility on electrophoresis at pH 6.5 and 3.5 as arginine hydrazide which had been prepared by incubation of arginine ethyl ester with equimolar hydrazine in dry methanol. The arginine hydrazide was purified by separation of the components of the reaction mix by electrophoresis at pH 6.5.

These results show that hydrazine had reacted exclusively with the α carboxyl of Arg-141α. No evidence was found for any proteolytic cleavage of hemoglobin by the trypsin treatment since sodium dodecyl sulfate electrophoresis in 15% gels (Weber and Osborn, 1969) showed the absence of fragments of less than 16 000 daltons.

Structure of Hydrazide Hemoglobin. The difference map of hydrazide hemoglobin superimposed on the 2.5-Å native map is shown in Figure 2. Major features of the native map are labeled in Figure 3, and Figure 4 is a sketch of deoxyhemoglobin in the region of Arg-141α_1.

In the difference map the pair of negative and positive peaks a and A show a shift of the ϵ amino of Lys-127α_2 away from the central cavity and toward Asp-6α_2. Similarly, the pair of negative and positive peaks b and B show a large movement of the hydrazide group away from the ϵ amino of Lys-127α_2. This movement causes the adjacent backbone atoms to move toward the central cavity as shown by the difference peaks c and C. In addition, some weak difference electron density peaks on adjacent sections (not shown) indicate a small movement of Tyr-140α, a slight loosening of the Arg-141α_1–Asp-126α_2 salt bridge, and a movement of the α-amino group of Val-1α_2 toward the guanidinium ion of Arg-141α_1. The latter movement, along with the removal of the negatively charged terminal carboxyl group causes an increase in the occupancy of the inorganic anion (labeled X^- in Figures 3 and 4) as shown by positive peak D. The absence of any other significant features in the difference map confirms the chemical specificity of the hydrazinolysis.

Oxygen Equilibrium Curves. Oxygen equilibrium curves of hydrazide hemoglobin and normal hemoglobin at 20

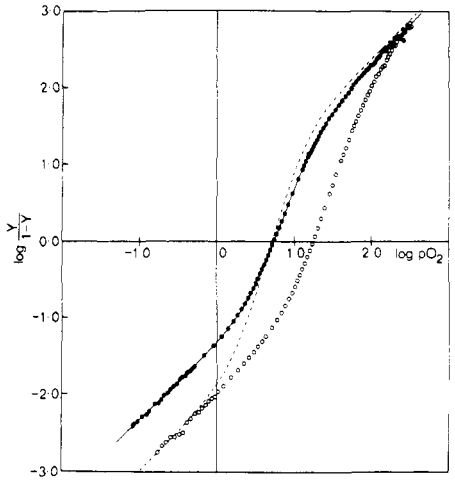

FIGURE 5: Points taken during the oxygen equilibrium curves of normal hemoglobin (○) and hydrazide hemoglobin (●) measured at 700 nm. Experimental conditions: hemoglobin concentration 20 mg/mL in 0.2 M Bistris, 0.1 M Cl^-, pH 6.8, at 25 °C. The lines show Hill plots calculated from the allosteric equation (Monod et al., 1965) with L = 27 000, K_R = 2.5 mm Hg^{-1}, and K_T = 0.045 mm Hg^{-1} (full line) or K_T = 0.01 mm Hg^{-1} (dashed line). The same full line was found when the change in K_T was confined to $K_{T\alpha}$ only, i.e. by making $K_{T\beta}$ = 0.01 mm Hg^{-1} and $K_{T\alpha}$ = 0.08 mm Hg^{-1}. The allosteric parameters calculated from the normal hemoglobin equilibrium curve were K_T = 0.01 mm Hg^{-1}, K_R = 3.4 mm Hg^{-1}, $L = 9.64 \times 10^6$.

mg/mL are shown in Figure 5. Curves were also measured at 10 mg/mL and 5 mg/mL also at 700 nm. The K_1 values for hydrazide hemoglobin measured from these curves were 0.041 (±0.003) mm Hg^{-1} at 20 mg/mL, 0.038 (±0.006) mm Hg^{-1} at 10 mg/mL, and 0.035 (±0.005) mm Hg^{-1} at 5 mg/mL. As in normal hemoglobin (Imai et al., 1970), no significant change in K_1 is detected over this concentration range, showing that the effect of dimers of K_1 is slight. Hence meaningful values of K_T can be calculated from these data. We first calculated L from K_R (equal to K_4 under these conditions) and log P_{50} (Edelstein, 1971), then computed a series of Hill plots using various values of K_T and finally compared them with the measured Hill plot in Figure 5. If K_T is left unaltered the observed curve cannot be fitted to a theoretical one without a large deviation at the bottom of the curve (Figure 5). Hence a substantial change in K_T must have occurred in hydrazide hemoglobin. The error in the K_T estimations would be the same as the error in the K_1 measurements shown above because under conditions of high cooperativity and protein concentration K_T is almost the same as K_1 (Ackers et al., 1975; Baldwin, 1975).

Removal of the hydrazide by incubation with trypsin completely regenerates the normal equilibrium curve (Kilmartin et al., 1975b).

The wavelength dependence of the equilibrium curves of hydrazide hemoglobin was not measured because of shortage of sample. In view of the lack of wavelength dependence of normal hemoglobin under these conditions (K. Imai, manuscript in preparation) any wavelength dependence in hydrazide hemoglobin would probably be slight.

Discussion

We have shown that trypsin catalyses the specific addition of hydrazine to the α carboxyl of Arg-141α of hemoglobin. X-ray analysis shows that the major structural change is the loss of the salt bridge between the α carboxyl of Arg-141α_1 and the ϵ amino of Lys-127α_2. The functional consequences are an increase in oxygen affinity and an increase in K_T. Since the difference Fourier synthesis shows no other major structural changes, this increase in K_T is most likely caused by the breakage of the Arg-141α_1-Lys-127α_2 salt bridge which specifically stabilizes the T structure. In line with this, the hydrazide equilibrium curve could equally well be fitted by confining the change in K_T to $K_{T\alpha}$ alone (Figure 5). It seems that with high cooperativity the position of the bottom asymptote is relatively insensitive to compensatory chain heterogeneity. The change in K_T is in agreement with Perutz' (1970) stereochemical mechanism and shows that this salt bridge is partially responsible for the free energy of cooperativity.

If the increased occupancy of the inorganic anion reflects a similar increase for Cl^- in solution, this would strengthen the T structure and partly compensate for the loss of the Arg-141α_1-Lys-127α_2 salt bridge. The loosening of the salt bridge between the guanidinium ion of Arg-141α_1 and Asp-126α_2 and the small movement of Tyr-140α appear to be secondary effects which are linked to the loss of the Arg-141α_1-Lys-127α_2 salt bridge. These structural changes are also in accord with the stereochemical pathway outlined by Perutz (1970), and may contribute to the observed increase in K_T. Hydrazinolysis does not significantly change K_R because the top asymptotes of the two equilibrium curves coincide (Figure 5). This agrees with the finding that the Arg-141α_1-Lys-127α_2 salt bridge is not found in the R structure (Heidner et al., 1976).

The trypsin catalyzed hydrazinolysis described in this paper should have general application, but an attempt to apply it to the α-carboxyl groups of the β chains was unsuccessful. We tried to activate the α carboxyl of His-146β in normal hemoglobin or of Tyr-145β in des-(His 146β)hemoglobin (Kilmartin et al., 1975a) by incubating the hemoglobins in 2 M hydrazine at pH 8.5 with chymotrypsin (1:10) at 40 °C for 4 h. After inhibition of chymotrypsin by *p*-methylsulfonyl fluoride and removal of hydrazine by gel filtration, we assayed the hydrazine released from the hemoglobin after further incubation with chymotrypsin. About 0.05 mol of hydrazine/$\alpha\beta$ dimer was released showing that formation of hydrazide was poor; also some proteolytic cleavage was evident from sodium dodecyl sulfate gels. So the hydrazinolysis of the α carboxyl of Arg-141α by trypsin is accelerated, probably because hemoglobin binds in a uniquely favorable way to trypsin allowing easy insertion of Arg-141α into the active site.

Acknowledgments

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