Amadori Rearrangement Potential of Hemoglobin at Its Glycation Sites Is Dependent on the Three-Dimensional Structure of Protein†

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ABSTRACT: The site selectivity of nonenzymic glycation of proteins has been suggested to be a consequence of the Amadori rearrangement activity of the protein at the respective glycation sites [Acharya, A. S., Roy, R. P., & Dorai, B. (1991) *J. Protein Chem. 10*, 345–358]. The catalytic activity that determines the potential of a site for nonenzymic glycation is the propensity of its microenvironment to isomerize the protein bound aldose (aldimine) to a protein bound ketose (ketoamine). The catalytic power of the microenvironment of the glycation sites could be endowed to them either by the amino acid sequence (nearest-neighbor linear effects) or by the higher order structure (tertiary/quaternary) of the protein (nearest-neighbor three-dimensional effect). In an attempt to resolve between these two structural concepts, the glycation potential of Val-1(α) and Lys-16(α), the residues of hemoglobin A exhibiting the least and the highest isomerization activity in the tetramer, respectively, has been compared in the segment α_{1-30}, isolated α-chain, and the tetramer. When α-chain is used as the substrate for the nonenzymic glycation, the influence of the quaternary structure of the tetramer will be absent. Similarly, the contribution of the tertiary and quaternary structure of the protein will be absent when α_{1-30} is used as the substrate. The microenvironment of Lys-16(α) exhibited hardly any Amadori rearrangement activity in the segment α_{1-30}. The tertiary structure of the α-chain induces a considerable degree of catalytic activity to the microenvironment of Lys-16(α) to isomerize the aldimine adduct at this site. The catalytic power of the microenvironment of Lys-16(α) is further enhanced by the quaternary structure of hemoglobin A. In contrast to the behavior of Lys-16(α), the catalytic activity of the microenvironment of Val-1(α) is reduced by the interaction of the quaternary structure of hemoglobin A almost by an order of magnitude. On the other hand, the loss of the tertiary structure of the α-chain reduced the catalytic activity of the microenvironment of Val-1(α) for nonenzymic glycation in a way similar to that seen with Lys-16(α). The perturbations of the local conformational features around Val-1(α) of hemoglobin A that occur on the removal of Arg-141(α) have very little influence on the catalytic activity at this microenvironment. The reactivity of Val-1(α) in horse hemoglobin for nonenzymic glycation is about five times higher than that of Val-1(α) of human hemoglobin. Val-1(α) is a part of the chloride binding site of hemoglobin A. The conformational elements of the chloride binding site of hemoglobin A are largely conserved in horse hemoglobin. The reactivity of Val-1(α) for nonenzymic glycation in des-Arg-141(α)-HbA and horse Hb establishes that the design principles of hemoglobin that contribute to the generation of a chloride binding site are distinct from the conformational features of the molecule that reduce the Amadori rearrangement activity of this site. The reactivity of Lys-16(α) in horse Hb and des-Arg-141(α)-hemoglobin A is nearly the same as that of human hemoglobin. The stereochemical features of this site are conserved well between the horse and human hemoglobin except for the replacement of Glu-116(α) by Asp in horse hemoglobin. The results thus establish that the Amadori rearrangement activity (catalytic power) of the glycation sites of hemoglobin A is a consequence of its three-dimensional structure rather than the amino acid sequence around the nonenzymic glycation site.

The posttranslational modification of proteins by nonenzymic glycation results in the derivatization of the amino groups of proteins by glucose as ketoamines (Bunn et al., 1975; Harding, 1985). Mechanistically, this reaction can be considered as the 2-oxo alkylation (Figure 1) of the amino groups by α-hydroxyaldehydes. However, this alkylation reaction involves the isomerization (Amadori rearrangement) of the initial reversible aldimine adduct (protein bound aldose) to a stable ketoamine (protein bound ketose). A very high degree of site selectivity is seen in the nonenzymic glycation reaction of proteins. Only a limited number of amino groups (mostly ϵ-amino groups of lysine residues) are derivatized in a given protein (Nigen & Manning, 1977; Acharya & Manning, 1980a; Garlick & Mazer, 1983; Watkins et al., 1985). However, the sites of derivatization of proteins by reductive alkylation with the same α-hydroxyaldehyde that also proceeds through the same aldimine intermediate (Figure 1) are distinct as compared with that of nonenzymic glycation (Acharya et al., 1983; Rogozinski et al., 1983; Watkins et al., 1985). Thus, the site selectivity of nonenzymic glycation appears to be a consequence of the Amadori rearrangement activity (catalytic power) of the nonenzymic glycation reactive sites of proteins.

Various suggestions have been made as to the factors that may facilitate the nonenzymic glycation of proteins. The possible role of His-2(β) in the higher reactivity of Val-1(β) of HbA[1] for nonenzymic glycation as compared to that of Val-1(α) has been suggested (Dixon, 1972; Acharya et al., 1983). Shilton and Walton (1991) have implicated the involvement

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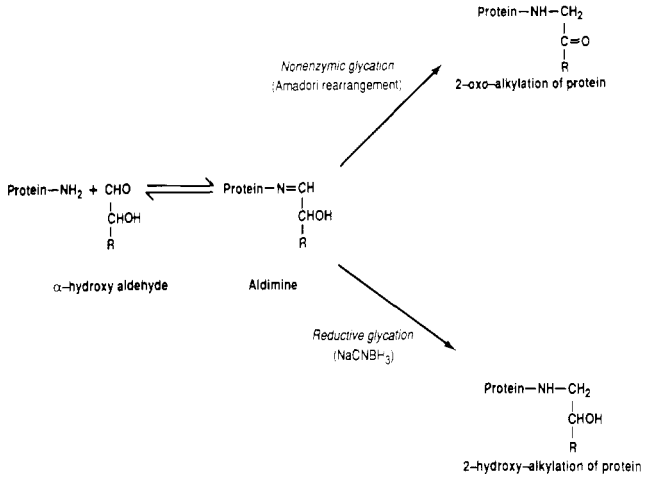

FIGURE 1: Two alternate modes of stabilization of protein bound aldimine adducts of α-hydroxyaldehydes.

of the active site histidine residues of alcohol dehydrogenase in the nonenzymic glycation of its active site Lys residues. The role of carboxyl groups that are proximal to the glycation sites in HbA in facilitating the Amadori rearrangement has been invoked by Shapiro et al. (1980). Iberg and Fluckiger (1986) have proposed that the lysine residues located in the Lys-Lys or Lys-His sequence of serum albumin are the potential sites for nonenzymic glycation. Watkins et al. (1987) postulated that the phosphate ions, bound to the phosphate binding region of the proteins like HbA and RNase A, can catalyze the Amadori rearrangement of the aldimines on the neighboring Lys residues.

Significant differences occur in the pK_a of the α- and ϵ-amino groups of proteins. Besides, it is also reasonable to anticipate significant variation in the pK_a of the reactive lysine residues of proteins (Matthew et al., 1979). Therefore, the extent of nonenzymic glycation of protein is unlikely to be correlated directly with the aldimine to ketoamine isomerization potential (Amadori rearrangement activity) of the microenvironment of the respective glycation sites. Accordingly, using the HbA–glyceraldehyde system, we have estimated the relative reactivity of the amino groups of HbA to undergo reductive alkylation and nonenzymic glycation (Acharya et al., 1991). From this data, a semiquantitative estimate of the relative catalytic power of the glycation sites (relative intrinsic Amadori rearrangement activity) was calculated. The catalytic power of the glycation sites of HbA decreased in the order Lys-16(α), Lys-82(β), Lys-66(β), Lys-61(α), Val-1(β), and Val-1(α). The catalytic power of the microenvironment of Lys-16(α) is nearly 3 orders of magnitude higher than that of Val-1(α) and 50 times higher than that of Val-1(β). Thus, it is clear that the rate of the isomerization reaction at each of the glycation prone sites of HbA is distinct.

The Amadori rearrangement activity at the individual glycation prone sites of HbA could come either from the primary structure (influence of neighboring amino acid residue in the sequence) or from the unique conformational aspects of the protein around the glycation sites (influence of the neighboring amino acid in the three-dimensional structure).

[1] Abbreviations: HbA, normal human hemoglobin; RP-HPLC, reverse-phase high-performance liquid chromatography; α- and β-chains, subunits of hemoglobin containing the noncovalently bound heme; PMB, *p*-(hydroxymercuri)benzoate; PBS, phosphate-buffered saline; DPG, 2,3-diphosphoglycerate; RNase A, bovine pancreatic ribonuclease A; TFA, trifluoroacetic acid; α_{1-30}, amino-terminal thirty-residue fragment of α-globin generated by the V8 protease digestion of the polypeptide chain.

In an attempt to resolve these structural concepts, the chemical reactivity of Lys-16(α) and Val-1(α) has been investigated in two other substrates, namely, the isolated α-chain (consequence of the loss of quaternary structural aspects) and the segment α_{1-30} (consequence of the loss of tertiary and quaternary structure). Lys-16(α) and Val-1(α) of HbA represent the sites that exhibit the highest and the lowest catalytic activity, respectively. The nonenzymic glycation studies with these substrates are expected to establish the influence of the three-dimensional structure of the protein on the chemical reactivity of the glycation prone sites. The contribution of subtle changes in the local conformational aspects of HbA on the chemical reactivity of these glycation prone sites has also been investigated by comparing the site selectivity of nonenzymic glycation of des-Arg-141(α)-HbA and horse Hb with that of HbA. The results of these investigations are presented here.

MATERIALS AND METHODS

Preparation of α_{1-30} Peptide. The general procedures for the preparation of lysate, purification of HbA, and isolation of the PMB α- and β-chains have been described previously (Acharya & Manning, 1980a). The globin, prepared from the purified hemoglobin A by acid acetone precipitation, was taken in 10 mM ammonium acetate (1 mg/mL), pH 4.0, and digested with *Staphylococcus aureus* V8 protease at 37 °C for 2 h and then lyophilized (Sahni et al., 1989). An enzyme to globin ratio of 1:200 (w/w) was employed for this digestion. The lyophilized sample was redissolved in 0.1% TFA and concentrated using Amicon concentrator using YM-10 membrane (Amicon Corp., Danvers, MA). Most of the undigested globin and larger peptides remained in the concentrator. The filtrate, containing the smaller peptides, namely, α_{1-30} and β_{1-22}, was lyophilized. α_{1-30} was purified by RP-HPLC using a semipreparative Aquapore RP-300 column. A linear gradient of 5–50% acetonitrile containing 0.1% TFA was used to elute the peptides. Identity of α_{1-30} was confirmed by the tryptic peptide mapping and amino acid analysis.

Preparation of Des-Arg-141(α)HbA. HbA (CO form, 10 mg/mL) was digested with carboxypeptidase B in 15 mM phosphate buffer, pH 8.0, at 25 °C for 3 h (Zito et al., 1964; Kilmartin et al., 1975). The ratio of enzyme to protein employed was 1:150. The extent of digestion was established from the free arginine content in a small aliquot (100 μL) of the reaction mixture. The protein in the aliquot was precipitated by adding 200 μL of the precipitant prepared by mixing 5 parts of 5% trichloroacetic acid and 3 parts of 0.2 M sodium citrate buffer, pH 2.2 (sodium diluent from Beckman). After the precipitated protein was separated by centrifugation, the supernatant was analyzed for arginine content with the Beckman amino acid analyzer, 126 AA. The concentration of arginine in a 3-h digest indicated that more than 90% of HbA has lost its carboxyl-terminal Arg residue of the α-chain. Therefore, a 3-h digest of the reaction mixture was dialyzed against 10 mM phosphate buffer, pH 6.0, and chromatographed on a CM-52 column (0.9 × 30 cm) that has been previously equilibrated with the same buffer. The protein was eluted using a linear gradient of 250 mL each of 10 mM phosphate buffer, pH 6.0, to 100 mM phosphate buffer, pH 6.0. The carboxypeptidase B digested component was isolated, concentrated, and dialyzed against PBS, pH 7.4.

Purification of Horse Hb. The lysate from horse erythrocytes (commercial sample, Pel-Freez) was dialyzed against three changes of 8 L each of PBS and then against another three changes of 6 L each of 50 mM Tris–acetate buffer, pH

8.5. The dialyzed sample was chromatographed on a DE-52 column (2.5 × 50 cm). The protein was eluted with a linear gradient of 1000 mL each of 50 mM Tris–acetate buffer, pH 8.0, to 50 mM Tris–acetate buffer, pH 7.0. At the end of the gradient (1800 mL), an additional 700 mL of final buffer was used to elute the protein completely. The protein eluting between 1860 and 1890 mL was pooled, concentrated, and dialyzed with PBS, pH 7.4, and used for the nonenzymic glycation studies, after saturating with carbon monoxide.

Nonenzymic Glycation of the Peptide and Protein Substrates. Carbonmonoxy forms of α-chain (2 mM), HbA, des-Arg-141(α)-HbA, and horse Hb, 1 mM each, were incubated separately with 10 mM [^{14}C]glyceraldehyde in PBS, pH 7.4, at 24 °C for 3 h. The reaction mixture was gel filtered on a column of Sephadex G-25 to separate the protein from the excess of the reagent. The extent of nonenzymic glycation was determined from the amount of the label incorporated into the protein (Acharya & Manning, 1980b). It has been previously demonstrated that the radioactive glyceraldehyde bound to HbA after the gel filtration represents the ketoamine adduct and the aldimine adducts do not survive this filtration step (Acharya & Manning, 1980b; Acharya & Sussman, 1984). α_{1-30}, at a concentration of 2 mM, was incubated with 10 mM [^{14}C]glyceraldehyde in PBS, pH 7.4, for 3 h at 24 °C. After the incubation, the peptide was separated from the excess reagent by RP-HPLC. The extent of modification was determined from the ^{14}C label incorporated into the peptide, the peptide concentration being quantitated by amino acid analysis.

Kinetics of Nonenzymic Glycation of HbA. One of the substrates used in the present study, i.e., the isolated α-chain, showed a time-dependent precipitation when incubated at 37 °C. In order to eliminate this complication, the nonenzymic glycation of α-chain and hence of the rest of the substrates had to be studied at the room temperature (24 °C) instead of 37 °C, the temperature that was used for our earlier studies. The kinetics of nonenzymic glycation of HbA with glyceraldehyde was studied at 24 °C to determine the influence of temperature on the rate of Amadori rearrangement and the site selectivity. Aliquots were drawn at different time intervals and gel filtered on a column of Sephadex G-25 (0.9 × 30 cm) and equilibrated and eluted with PBS to separate the protein from the excess reagent. The amount of glyceraldehyde incorporated into the protein represents the extent of nonenzymic glycation (Acharya & Manning, 1980b). The nonenzymic glycation of the protein was linear at least during the initial stages of the reaction just as the reaction studied at 37 °C (Acharya et al., 1991). However, a 4-fold decrease in the rate of glycation was observed when the temperature was lowered from 37 to 24 °C. On the other hand, the site selectivity of the glycation of HbA at 24 °C remained nearly the same as that at 37 °C (Table I). The relative distribution of modification at each of the highly reactive glycation sites is nearly the same at both temperatures (Table I), even though the extent of modification in the sample glycated at 24 °C is only 50% of that sample glycated at 37 °C. Comparison of the site selectivity of reductive and nonenzymic glycation at 24 °C (data not shown) has confirmed that even at this lower temperature the Amadori rearrangement is the rate-limiting step of the nonenzymic glycation just as at 37 °C (Acharya et al., 1983). Besides, the site selectivity of reductive glycation also remained essentially unchanged. Thus, the influence of the temperature on the rate of Amadori rearrangement of the aldimines at all the sites appears to be nearly the same.

Table I: Influence of Temperature on the Site Selectivity of Nonenzymic Glycation of HbA

glycation site	% modification[a] 24 °C	37 °C
Val-1(α)	2.2	2.7
Val-1(β)	22.5	23.0
Lys-16(α)	32.3	38.5
Lys-61(α)	6.2	7.0
Lys-66(β)	13.8	12.2
Lys-82(β)	8.4	7.0

[a] The nonenzymic glycation of HbA (1 mM) was carried out with 10 mM glyceraldehyde at pH 7.4, for 3 h at 24 °C and 90 min at 37 °C. Under these conditions, 1.4 and 2.6 mol of glyceraldehyde/mol of tetramer were incorporated at 24 °C and 37 °C, respectively.

Reconstitution of the Tetramer from Glycated α-Chain. To avoid the precipitation of nonreductively modified α-chain during sodium borohydride reduction (to convert the ketoamine derivative to 2,3-dihydroxypropyl derivative), it was found necessary to reconstitute the modified chain into tetramer by hybridizing with the complementary β-chain before reduction. The glycated PMB α-chain (after gel filtration of the reacted sample to get rid of the excess reagent) and the unglycated PMB β-chain were taken in a ratio of 1:1.1 in 50 mM phosphate buffer, pH 7.4, and incubated with 100 mM 2-mercaptoethanol for 2 h at 4 °C. The hybridized sample was concentrated and gel filtered on a column of Sephadex G-25 and equilibrated and eluted with PBS.

Tryptic digestion of glycated samples and the peptide mapping were carried out as described previously by Acharya et al. (1991).

RESULTS

Nonenzymic Glycation of α-Chain and α_{1-30}. In HbA, Val-1(α) represents the least reactive nonenzymic glycation site, while the Lys-16(α) is the most reactive site (Acharya & Manning, 1980a). In an attempt to establish the contribution of the conformational aspects of HbA in endowing the respective sites with the Amadori rearrangement activity, the reactivity of the amino groups of α-chain and α_{1-30} for nonenzymic glycation has been investigated. In these two substrates, the primary structure of the protein around the respective two glycation sites is conserved, but the conformational aspects of the microenvironments are expected to be perturbed to different degrees as compared to that present in the tetramer. α_{1-30} has been shown to exhibit very little secondary structure under physiological conditions (Acharya et al., 1992). On the other hand, the α-chain has well-defined secondary and tertiary structures, but it lacks the quaternary interactions of the tetramer. The nonenzymic glycation of α-chain at 24 °C for 3 h incorporated 0.35 mol of glyceraldehyde/mol of the chain (Table II). Under similar conditions, 1.4 mol of glyceraldehyde were incorporated into 1 mol of HbA (0.7 mol/αβ dimer), and nearly 50% of that was present in the α-chain. Thus, the extent of modification of isolated α-chain is comparable to that of α-chain of the intact protein ($\alpha_2\beta_2$ structure). However, it should be noted here that during the nonenzymic glycation of isolated α-chain, there is no β-chain in the glycation system, and hence the molar ratio of glyceraldehyde to amino groups of protein is doubled as compared to that in HbA glycation system. We have shown earlier with HbA (Acharya et al., 1991) and more recently with RNase A (Acharya et al., manuscript in preparation) that at these concentrations of glyceraldehyde the extent of glycation is directly proportional to the concentration of aldose.

Table II: Influence of Structure of the Substrate on Nonenzymic Glycation

substrate	nonenzymic glycation in the region (mol of aldose/mol of substrate)		
	$\alpha_2\beta_2$	α-chain	α_{1-30}
HbA	1.400	0.343[a]	0.241[b]
α-chain		0.350	0.217[b]
α_{1-30}			0.014

[a] This is the sum of modification of Val-1(α), Lys-16(α), Lys-61(α), and one of the unidentified sites of HbA which was also found in the modification of isolated α-chain. [b] This is the sum of the modification of Val-1(α) and Lys-16(α) in the respective substrates.

Table III: Influence of Tertiary/Quaternary Structure of HbA on the Reactivity of Glycation Sites of α-Chain[a]

glycation site	HbA (mol of aldose/ mol of site)	α-chain (mol of aldose/ mol of site)	α(1–30) (mol of aldose/ mol of site)
Val-1(α)	0.015	0.137	0.007
Lys-16(α)	0.226	0.080	0.002
Lys-61(α)	0.043	0.032	

[a] The reactions at the individual glycation sites were calculated from the radioactivity associated with respective tryptic peptides in the RPHPLC maps of the glycated samples.

Thus, one would expect nearly double the amount of modification in the isolated α-chain as compared to that of α-chain in the intact tetramer.

On nonenzymic glycation of the segment α_{1-30}, at 24 °C for 3 h, 0.014 mol of glyceraldehyde/mol of peptide was incorporated. Under identical conditions, nearly 0.241 mol and 0.217 mol of glyceraldehyde were incorporated into the α_{1-30} region when this segment was subjected to nonenzymic glycation as a part of HbA or of the isolated α-chain, respectively (Table II). Again, it should be noted that the molar ratio of glyceraldehyde to amino groups during the glycation of isolated segment α_{1-30} is significantly higher as compared with that in the case of HbA or of α-chain reactions. In spite of this kinetic advantage, the amino groups of α_{1-30} exhibit hardly any reactivity toward nonenzymic glycation. The loss of tertiary and quaternary structure of the protein has abolished, almost completely, the chemical reactivity of Lys-16(α) and Val-1(α) of HbA for nonenzymic glycation. The reductive glycation of α_{1-30} revealed that in spite of the fact that the molar ratio of aldotriose to the amino groups of the substrate has increased, the reductive glycation of peptide by itself decreased by nearly 50%. However, the nonenzymic glycation was reduced by nearly 17-fold, clearly demonstrating the influence of the tertiary/quaternary structure of HbA in endowing the Amadori rearrangement potential to the glycation prone amino groups of α_{1-30}.

Site Selectivity of Nonenzymic Glycation of α-Chain and α_{1-30}. Besides the reduction in the nonenzymic glycation of the amino groups in the isolated chain, the loss of the quaternary structure could also influence the site selectivity of the derivatization process. In an attempt to establish whether indeed this has occurred, the tryptic peptide map of nonenzymically glycated α-chain has been generated and the site selectivity of glycation has been established (Table III). The loss of the quaternary structure resulted in a significant change in the site selectivity of the nonenzymic glycation of the chain. The reactivity of Lys-16(α) is lowered by nearly 60% in the isolated α-chain as compared to that in HbA (without normalizing for the glyceraldehyde concentration). The reactivity of Lys-61(α), one of the minor sites of glycation, is reduced only by about 25%. Nonetheless, the propensity of these two amino groups to undergo reductive glycation is nearly the same as that in the tetramer. Therefore, the results establish the functional role of quaternary structure of HbA in enhancing the Amadori rearrangement potential of the microenvironment of Lys-16(α) and Lys-61(α) in HbA.

In contrast to the decreased reactivity of Lys-16(α) and Lys-61(α), the reactivity of Val-1(α) increased nearly 10-fold in the isolated α-chain as compared to that in HbA. The reactivity of Val-1(α) is nearly twice that of Lys-16(α) and four times that of Lys-61(α). Thus, in the isolated α-chain, Val-1(α) is the most reactive nonenzymic glycation site, as opposed to its being the site of least reactivity in the tetramer. The quaternary interactions of HbA around Val-1(α) apparently decrease the Amadori rearrangement activity of the microenvironment of Val-1(α). However, it should be noted here that this reduction in the reactivity is not a consequence of the stereochemical factors limiting the accessibility of the site to the reagent (glyceraldehyde) since the reactivity of Val-1(α) for reductive glycation with glyceraldehyde in the tetramer is comparable to that of Val-1(β) and the nonenzymic glycation at Val-1(β) in the tetramer is nearly 10 times that at Val-1(α) (Acharya et al., 1983). The reactivity of Val-1(α) to generate the aldimine in the isolated α-chain has been found to be nearly twice that in the tetramer, presumably a consequence of the higher molar ratio of aldose to amino groups present during the reaction of the isolated α-chain. Therefore, though a part of the increased nonenzymic glycation of Val-1(α) in the isolated chain could be attributed to the increase in the amount of the aldimine formation at this site, a significant amount of the increase in the nonenzymic glycation appears to be the consequence of the loss of the quaternary structure.

Lys-16(α) of the isolated α_{1-30} exhibits hardly any reactivity toward nonenzymic glycation even though this residue is the most reactive site in the tetramer, and retains a considerable degree of its reactivity in the isolated α-chain (Table III). Thus, the Amadori rearrangement potential of Lys-16(α) seen in the tetramer (Acharya et al., 1991) is endowed to it by the tertiary/quaternary structure of HbA, and the tertiary structure of the α-chain makes the major contribution. The reactivity of Val-1(α) is also reduced considerably in the isolated segment α_{1-30} as compared to that in the isolated α-chain (Table III). The chemical reactivity of Lys-16(α) and Val-1(α) of α_{1-30} for reductive glycation is nearly 70% and 90%, respectively, of the same residue in the tetramer and 70% and 60%, respectively, of that in the isolated chain. The results demonstrate that the tertiary and the quaternary structural elements of the protein enhance the reactivity of these residues for nonenzymic glycation by modulating the Amadori rearrangement activity of the microenvironment of these sites. The linear amino acid sequence of this region appears to have a limited contribution toward the Amadori rearrangement potential of the sites.

Nonenzymic Glycation of Des-Arg-141(α)-HbA. The comparison of the reactivity of Val-1(α) in the isolated α-chain and the tetramer, immediately, suggests that the subunit interactions of the tetramer contribute to the reduction of the chemical reactivity of Val-1(α) of isolated chain. In the tetramer, the carboxyl-terminal Arg-141 of one α-chain is brought to the proximity of the amino-terminal Val-1 of the other α-chain (Muirhead & Greer, 1970; Fermi, 1975). These two residues together form part of the chloride ion binding site of the molecule (O'Donnell et al., 1979). Modification or removal of either one of these residues influences the oxygen affinity of HbA and also decreases the sensitivity of the O_2

Table IV: Site Selectivity of Nonenzymic Glycation[a] of Des-Arg-141(α)-HbA

glycation site	HbA (mol of aldose/mol of site)	des-Arg-141(α)-HbA (mol of aldose/mol of site)
Val-1(α)	0.015	0.017
Val-1(β)	0.158	0.148
Lys-16(α)	0.226	0.212
Lys-61(α)	0.043	0.042
Lys-66(β)	0.097	0.086
Lys-82(β)	0.059	0.077

[a] The distributions of glycation at the various peptides of the protein were calculated from the RPHPLC maps of the tryptic digest of the sample.

affinity of the molecule to chloride ions (O'Donnell et al., 1979; Kilmartin et al., 1975, 1977; Nagai et al., 1982). The reduced reactivity of Val-1(α) for nonenzymic glycation in HbA as compared to that in isolated α-chain may be a reflection of the role for one or both of the functional (carboxyl and guanidino) groups of Arg-141(α) to reduce the Amadori rearrangement activity at this site. The nonenzymic glycation of des-Arg-141(α)-HbA has been investigated and compared with that of HbA to delineate the possible role of this residue. The reaction of this derivative of HbA with glyceraldehyde for 3 h at 24 °C resulted in an incorporation of about 1.6 mol of aldose/mol of the protein which is comparable to that incorporated into HbA (1.4 mol), under similar conditions. Thus, the overall reactivity of the protein is not influenced significantly by the removal of Arg-141(α) from the protein. Besides, the site selectivity of glycation of this derivative is also nearly the same as that of HbA (Table IV). Particularly, the reactivity of Val-1(α) remained almost the same in both substrates indicating that the reduction of the Amadori rearrangement potential of this site is not a consequence of the presence of Arg-141(α) in HbA. Thus, it is clear that the quaternary structural features of HbA that result in the generation of the chloride ion binding site are not the ones that dictate the Amadori rearrangement potential of Val-1(α).

Nonenzymic Glycation of Horse Hb. The results presented so far have clearly established that the chemical reactivity of Lys-16(α) and Val-1(α) of HbA toward nonenzymic glycation is determined by the quaternary structure of the protein. A study of the reactivity of these residues in other hemoglobins with a structure similar to that of HbA could provide new insights into the mechanism that dictates site selectivity of glycation. The overall quaternary structural features of HbA are conserved in horse Hb, in spite of multiple site mutations in both α- and β-chains (Bolton & Perutz, 1970; Ladner et al., 1977). The nonenzymic glycation of horse Hb with glyceraldehyde at 24 °C for 3 h resulted in an incorporation of about 1.6 mol of glyceraldehyde/mol of protein, which is comparable to that of HbA (1.4 mol of aldose/mol of protein). The tryptic peptide map of glycated horse Hb (Figure 2A) indicated that the chemical reactivity of Lys-16(α) toward nonenzymic glycation is comparable in both the hemoglobins. The site selectivity of reductive glycation of horse Hb is also comparable to that of HbA (data not shown). These results thus suggest that the Amadori rearrangement potential at Lys-16(α) of these two proteins is nearly the same.

In contrast to the situation seen with Lys-16(α), the chemical reactivity of Val-1(α) of horse Hb is about 5 times higher than that of Val-1(α) of human Hb. As noted earlier, the site selectivity of horse Hb for reductive alkylation with glyceraldehyde is comparable to that of HbA. Therefore, the enhanced reactivity of Val-1(α) of horse Hb suggests that the

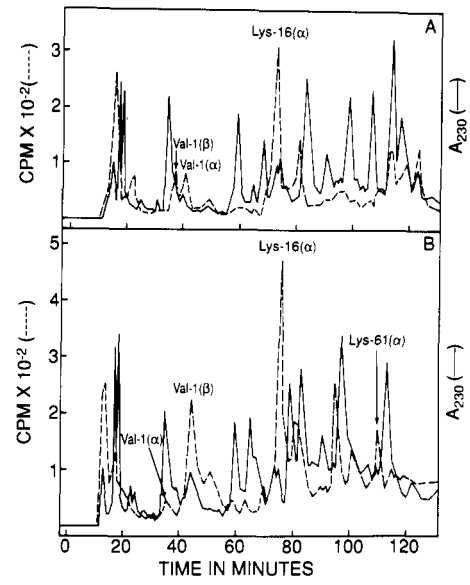

FIGURE 2: Tryptic peptide map of nonenzymically glycated horse Hb (A) and HbA (B). The horse Hb/HbA (1 mM) was incubated with 10 mM [^{14}C]glyceraldehyde at pH 7.4 and 24 °C for 3 h. After being digested with trypsin, the samples (~7 mg of horse Hb and 10 mg of HbA) were chromatographed on RP-HPLC using an RP-300 semipreparative column and a gradient of 5–50% acetonitrile (0.1% TFA) in 160 min. The specific activity of [^{14}C]glyceraldehyde was 913 and 1090 cpm/nmol for horse Hb and HbA, respectively.

microenvironment of Val-1(α) is endowed with a higher isomerization potential compared with that of HbA. Thus, these two hemoglobins are distinct with regard to their conformation around Val-1(α), at least as reflected in the Amadori rearrangement potential of this domain. Although the overall quaternary structure of the two hemoglobins has been suggested to be similar, there are 42 replacements per αβ dimer in horse Hb, as compared to human Hb. One or more of these mutations have apparently created subtle differences in the microenvironment of Val-1(α), which has contributed to the lowering of quaternary structural influence of the tetrameric molecule to reduce the Amadori rearrangement activity of the microenvironment of this residue, as compared with the isolated human α-chain. The conformational aspects of the chloride ion binding region of horse Hb and HbA are comparable. Thus, the differential reactivity of Val-1(α) of these two hemoglobins indicate that factors other than the one that modulate the chloride ion binding determine the reactivity of Val-1(α) for nonenzymic glycation. This conclusion is consistent with the results obtained with des-Arg-141(α)-HbA.

DISCUSSION

The reaction of glyceraldehyde with proteins has served as a "prototype" reaction to pursue the mechanistic details of the nonenzymic addition of aldose to proteins (Acharya & Susman, 1984; Acharya et al., 1983, 1989). In the present study, we have used the reaction of glyceraldehyde with Hb to establish whether the Amadori rearrangement activity of the glycation prone sites is dependent on the primary sequence of the protein (nearest-neighbor linear effect) or a consequence of the nearest-neighbor stereochemical effect (conformational aspects). The study of the chemical reactivity of Lys-16(α) and Val-1(α) in the isolated α-chain has clearly established that the Amadori rearrangement potential of the glycation prone sites of HbA is predominantly a consequence of unique stereochemical arrangement of the residues in the vicinity of the glycation sites. The primary structure around the site has very little,

α-chain HbA : Val-Leu-Ser-Pro-Ala-Asp-Lys-Thr-Asn-Val
Horse Hb : Val-Leu-Ser-Ala-Ala-Asp-Lys-Thr-Asn-Val

β-chain HbA : Val-His-Leu-Thr-Pro-Glu-Glu-Lys-Ser-Ala
Horse Hb : Val-Gln-Leu-Ser-Gly-Glu-Glu-Lys-Ala-Ala

FIGURE 3: Comparison of the amino acid sequence of the first 10 residues of α- and β-chains of human and horse hemoglobins. The amino acid residues that are different between the two hemoglobins are boxed in.

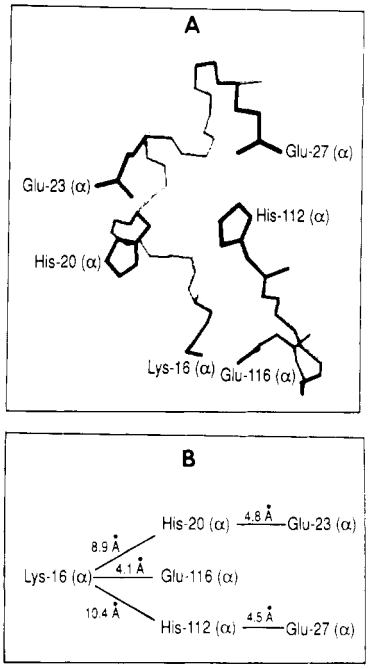

FIGURE 4: Stereochemistry of the side chain of basic and acidic amino acid residues present in the microenvironment of the ε-amino group of Lys-16(α) of HbA. (A) A computer image taken from the files of Protein Data Bank (Fermi & Perutz, 1984). Two fragments of α-chain, containing the residues 16–27 and 112–116 of the α-chain, are displayed. The side chains of unwanted residues are deleted for clarity of the disposition of the acidic and the basic side chains. (B) The interresidue distances of the respective residues taken from the same file. The atoms from which the interresidue distance was measured is as follows: ε-nitrogen in Lys-16(α), C-2 of imidazole of His-20 and -112 of the α-chain, and γ-carboxyl carbon in Glu-23, -27, and -116 of the same chain.

if any, dictative role in endowing the region with an aldimine to ketoamine isomerization potential.

The chemical reactivities of Val-1(α) and Val-1(β) of horse Hb for nonenzymic glycation are nearly the same (Figure 2A). This equal reactivity of Val-1(α) and Val-1(β) of horse Hb is distinct from that seen with HbA. At least two molecular events could have contributed to this identical reactivity of Val-1(α) and Val-1(β). On one hand, the reactivity of Val-1(α) has increased by about 5-fold and on the other hand the reactivity of Val-1(β) decreased by about 2-fold as compared to that in HbA. His-2(β) of HbA is replaced by a Gln residue in horse Hb (Figure 3). The lower reactivity of Val-1(β) in horse Hb, compared to that in HbA, could certainly be argued as a reflection of the possible role of His-2(β) in the nonenzymic glycation of Val-1(β) (nearest-neighbor linear effects). However, the reactivity of Val-1(β) is not completely abolished in horse Hb; i.e., this residue does not function as the unique catalytic residue for the Amadori rearrangement activity of the microenvironment of Val-1(β) of HbA. Val-1(β) and His-2(β) form a part of the DPG binding pocket of the protein, which indeed involves the constellation of eight positive charges contributed by the two β-chains of HbA. The reduction in the Amadori rearrangement potential around Val-1(β) in horse Hb could therefore be a consequence of the reduction in the overall net positive charge of the microenvironment of this glycation site as a consequence of the mutation of His-2(β) to Gln, rather than being a reflection of sequence dependence of site selectivity. It should also be noted here that Pro-5(β) of HbA is mutated to Gly in horse Hb. It is conceivable that the increased flexibility of this amino-terminal segment in horse Hb as compared to its counterpart in HbA could also contribute to the differential chemical reactivity of Val-1(β) in the two proteins.

The modulation of the nonenzymic glycation by conformational aspects of the protein has been invoked previously. Several investigators (Smith et al., 1982; Roberts et al., 1984; Watkins et al., 1987; Bai et al., 1989) have demonstrated that the presence of DPG enhances the extent of nonenzymic glycation of HbA/model peptides, and this influence is pronounced in the deoxy conformation. These observations clearly reflect that the conformational aspects of the protein dictate the nonenzymic glycation. However, in none of these studies has any attempt been made to determine the particular step of the nonenzymic glycation reaction (aldimine formation versus the Amadori rearrangement) that is influenced by the conformational aspects. The present study clearly distinguishes itself from the earlier studies in that it establishes, for the first time, the strong contribution of the tertiary and the quaternary structural aspects of HbA dictating the catalytic power: i.e., the aldimine to ketoamine isomerization potential (triose phosphate isomerase or glucose isomerase type of catalytic activity) of the nonenzymic glycation sites of HbA.

The increase in the reactivity of Val-1(α) in horse Hb compared to in HbA cannot be explained simply on the basis of the primary structure of the two proteins. The amino acid sequence around Val-1(α) is nearly the same in both hemoglobins except for Pro-4(α) of HbA, which is mutated to Ala in horse Hb (Figure 3). This Pro to Ala mutation could increase the flexibility of this segment in horse Hb, which may be responsible for the increase in the Amadori rearrangement activity of this site. The reduction of the glycation potential of Val-1(α) of the chain by the quaternary structure of Hb appears to be less efficient in horse Hb as compared to that in HbA.

Thus, it is clear that the amino acid sequence of the protein is not the primary determinant of the site selectivity of its nonenzymic glycation. Accordingly, one could speculate that some generality exists in the conformational features of glycation prone sites of various proteins since a common catalytic step is involved in these reactions. The precise nature of this "nonenzymic glycation motif" apparently determines the Amadori rearrangement potential and therefore the site selectivity of the nonenzymic glycation of proteins. Some of the glycation sites of HbA are located within the DPG binding pocket of the protein (Acharya & Manning, 1980a; Shapiro et al., 1980), and the glycation prone sites of RNase A are located in the active site region of the enzyme. Both of these regions are the proton-rich domains of the respective proteins. Thus, the Lys residues located in a proton-rich domain of a protein, and accessible for aldimine formation with the α-hydroxyaldehydes, may be considered as the first approximate description of a "structural motif" of the nonenzymic glycation prone sites of proteins.

Lys-16(α) is in close proximity to His-20(α) and His-112(α) in the three-dimensional structure of HbA and certainly satisfies the basic structural requirements proposed above for

a "nonenzymic glycation motif" (Figure 4A). Glu-23(α) is proximal to His-20(α) and Glu-27(α) is proximal to His-112(α), and these two acidic residues appear to serve as the anchoring points to retain a defined stereochemistry for the respective His residues. If one of the above histidine residues acts as a base catalyst in the Amadori rearrangement of Lys-16(α), the proximal Glu residue can stabilize the positive charge developed on the respective His residue. The interatomic distances between these residues in the crystal structure of HbA are shown in Figure 4B. It should also be noted that Lys-16(α) is located near the junction of the A and B helices of the chain. The amino terminus of the B-helix of the chain is expected to have half of a positive charge and the carboxyl terminus of the A-helix is expected to have half of a negative charge as a consequence of the macrodipole of the helix (Hol et al., 1978). In addition, Glu-116(α) is also proximal to Lys-16(α). Thus, the microenvironment around Lys-16(α) represents a region of positive and negative charges with an overall net positive charge. These stereochemical aspects of the microenvironment of Lys-16(α) are largely conserved in horse hemoglobin except for the substitution of Glu-116(α) by an Asp residue. One or more of these residues and their geometry in the region has contributed to the Amadori rearrangement activity at Lys-16(α). Therefore, it will be of interest to elucidate the influence of site-specific mutations of His-20(α), His-112(α), and Glu-116(α) of HbA on the nonenzymic glycation of Lys-16(α).

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Registry No. Val, 72-18-4; Lys, 56-87-1; HbA, 9034-51-9.