The Effect of Hemoglobin Ligands on the Kinetics of Human Hemoglobin A₁c Formation*

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HbA₁c is the most prevalent of the minor human hemoglobins. It is formed by the nonenzymatic addition of glucose to the α-amino group of the β chain by an initial condensation reaction and a subsequent intermolecular Amadori rearrangement. We have developed a method of analysis which utilizes high performance liquid chromatography to follow the formation of HbA₁c and greatly simplifies the determination of the kinetic parameters associated with this reaction. This has allowed us to study the effects of several Hb ligands, including the hydrogen ion, on the kinetics of this glycosylation reaction.

Both the initial condensation reaction and the subsequent rearrangement are shown to exhibit acid catalysis, but the rate of the condensation step is limited by the extent of protonation of the α-amino group. The variation in kinetic parameters as a function of hydrogen ion concentration has allowed us to determine the probable reaction mechanism of HbA₁c formation by comparison to previously reported model systems of Schiff base formation and Amadori rearrangement. The formation of pre-HbA₁c from deoxy-Hb shows an increased forward rate when compared to oxy-Hb. The presence of physiologic concentrations of CO₂ causes a proportional decrease in both k₁ and k₋₁. 2,3-Diphosphoglycerate causes a significant increase in the k₆ of the formation reaction. The effects of CO and the substitution of L-glucose for D-glucose are not significant.

Human hemoglobin A₀ is nonenzymatically glycosylated in vivo at several sites (1). The most favored of these sites is the amino group of the amino-terminal valine of the β chain. To date, four distinct species of hemoglobin glycosylated at this site have been identified (2). The most abundant of these species is HbA₁c, which is formed by the addition of glucose to the α-amino group of the β chains (3). HbA₁c is homogeneous when analyzed by ion-exchange chromatography, but may be chemically separated into two isomers. As is shown in Fig. 1, the initial reversible condensation of glucose and hemoglobin forms an aldime or Schiff base which is able to undergo a nearly irreversible intermolecular Amadori rearrangement (3, 4). The Schiff base is known as labile HbA₀, or pre-HbA₁c and the ketoamine as stable HbA₁c, or simply HbA₁c (5).

Studies of the kinetics of HbA₁c formation have been stimulated by the observation that levels of this molecule are significantly elevated in persons with diabetes mellitus in proportion to their average blood glucose levels. An understanding of the HbA₁c structure and its formation has made its measurement a cornerstone in the assessment of long-term diabetes control (6).

In addition to the clinical utility of HbA₁c, it may also serve as a model for the study of the chemical and allosteric properties of the hemoglobin molecule. The α-amino group of the β chain is intimately involved in the normal physiologic function of hemoglobin. This group lies in the central cleft of the molecule adjacent to the 2-fold axis of symmetry and is directly involved with the binding of 2,3-DPG, CO₂, and hydrogen ions, and its position is altered by the binding of oxygen (7, 8).

The kinetics of HbA₁c formation have been studied by Higgins and Bunn (9) utilizing radiolabeled glucose and chemical modification of reaction intermediates and by Mortensen and Christophersen (10) by isoelectric focusing. Both of these studies examined a limited set of experimental conditions which we have been able to extend by developing a mathematical analysis which simplifies the determination of the kinetic parameters of HbA₁c formation. This has allowed us to investigate the effects of hydrogen ion concentration, 2,3-DPG, CO₂, CO₂, and hemoglobin oxygenation on the kinetics of HbA₁c formation. In our experiments the formation of HbA₁c is monitored by cation-exchange HPLC. The results of these experiments provide insight into the chemical mechanism of HbA₁c synthesis as well as on the effects of the ligands which physiologically interact with hemoglobin.

EXPERIMENTAL PROCEDURES

Red cell hemolysates were prepared as previously described (11). HbA₀ and minor glycosylated hemoglobin species were purified by the method of McDonald et al. (2). The purified HbA₀ was dialyzed overnight versus two changes of deionized distilled water and stored at 4°C.

HbA₀ was synthesized in a reaction mixture containing HbA₀ at approximately 0.2 mM (concentration of αβ dimer), D-glucose at approximately 110 mM, and potassium phosphate buffer at 50 mM in a volume of 500 μL. Following mixing at 4°C, the pH was adjusted to that required for the experiment (±0.01 pH units) by the addition of 2-μL aliquots of 0.5 M KH₂PO₄ or K₂HPO₄. Samples of the mixture were then removed for analysis of total hemoglobin and glucose concentrations. The reaction was initiated by raising the temperature to 37°C. In experiments designed to measure k₁ and k₋₁, the concentrations of HbA₀ and total HbA₁c (pre-HbA₁c plus stable HbA₁c) were determined by cation-exchange HPLC as previously described (11, 12). Aliquots of the reaction mixture were analyzed at time intervals so that four to five analyses could be performed before the reaction reached equilibrium. One or two analyses were then performed at

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‡ The abbreviations used are: 2,3-DPG, 2,3-diphosphoglycerate; HPLC, high performance liquid chromatography.
equilibrium. The in vitro synthesized HbAlc coeluted with HbA1c isolated from human erythrocytes.

Total hemoglobin concentration was measured by absorbance at 415 nm and expressed as the concentration of αβ dimer. The concentrations of individual hemoglobin species could then be calculated by knowing the percentage of each species from HPLC analysis. Glucose concentrations were measured on a Beckman glucose analyzer 2 (Beckman Instruments, Inc., Fullerton, CA).

In experiments designed to measure $k_b$, the reaction between glucose and HbA was first allowed to reach equilibrium. Then the slow linear increase in total HbA was monitored by HPLC at 10-24 h intervals for 50-100 h (see Fig. 2). In these much longer experiments the reaction mixture was sterilized by filtration through Millipore filter SLM0250S (Millipore Corp., Bedford, MA) immediately prior to the initiation of the experiment to avoid bacterial contamination.

All experiments were performed under air unless otherwise noted. In experiments performed under gasses other than air, 60-μl aliquots of the reaction mixture were gassed following the adjustment of the total mixture to the appropriate pH. In experiments conducted under 5% CO₂, the pH was adjusted immediately following gassing of the sample. Deoxyhemoglobin experiments were performed under 100% N₂.

In experiments involving 2,3-DPG, this species was added to the standard reaction mixture at the normal physiologic concentration of 4.2 mM (13). 2,3-DPG and I-glucose were purchased from Sigma. The two-tailed t test was used as a measure of statistical significance.

**Determination of Kinetic Parameters**—The aim of our experiments was to determine the kinetic parameters of HbAlc formation ($k_b$, $k_1$, and $k_2$) under a variety of conditions. The experimental design, simply stated, involved the mixing of HbA with glucose and then monitoring the formation of total HbAlc (pre-HbAlc plus stable HbAlc). For the purpose of this analysis, the time course of the synthesis of HbAlc may be divided into the two periods shown in Fig. 2. During the first phase of the reaction, the equilibrium concentration of pre-HbAlc is rapidly attained, and formation of stable HbAlc is minimal. Therefore, when measuring total HbAlc during this time period, we essentially monitored the isolated formation of pre-HbAlc. This allows the kinetics of pre-HbAlc formation to be studied in virtual isolation.

It is of concern that stable HbAlc formation during the initial phase of the experiment may not be negligible. The conditions under which stable HbAlc forms the largest proportion of total HbAlc during the approach to pre-HbAlc equilibrium are those of greatest $k_b$ and the slowest approach to equilibrium. Under these conditions pre-HbAlc is available to rearrange to stable HbAlc, and it has a longer period of time in which to do so before pre-HbAlc equilibrium is attained. Both of these conditions occurred in experiments conducted at pH 7.60. The rate constants from these experiments were used to generate Fig. 2. Under these conditions, stable HbAlc is calculated to form less than 0.5% of the total hemoglobin and less than 4% of the total HbAlc. Pre-HbAlc equilibrium is attained at approximately 9 h.

The data necessary for determining $k_b$ and $k_1$ during the first phase of HbAlc synthesis are the initial, several intermediate, and the equilibrium concentrations of HbAlc, HbA, and glucose. It should be noted that the concentration of glucose is essentially a constant throughout the entire experiment. Glucose is present at a concentration of approximately 110 mM, and the maximal concentration of total HbAlc is only 20 μM. Thus, only 0.02% of the total glucose combines with hemoglobin.

In the second phase of the experiment, pre-HbAlc rearranges to form stable HbAlc, and accumulates linearly with respect to time. The concentration of pre-HbAlc is essentially constant during this period so that any increase in total HbAlc is due to the formation of the stable adduct. The data necessary for the determination of $k_b$ are the equilibrium concentration of pre-HbAlc and the slope of the linear portion of the total HbAlc formation curve.

The following derivations provide the mathematical bases for the analysis of experimental data. Relations are obtained in which all variables may be experimentally determined and linear plots of the data constructed. The definition of variables as follows: [pre-HbAlc] = $X$, $k_1$ = concentration of pre-HbAlc; [HbA] = [HbAlc] = concentration of stable HbAlc; [G] = concentration of glucose; [A₀] = concentration of HbA₀, subscript ₀ = initial condition; and subscript $t$ = equilibrium condition.

**Equation 7**

$$\frac{d[\text{pre-HbAlc}]}{dt} = k_b[G][\text{HbA}] - k_1[\text{pre-HbAlc}].$$

(1)

Let $[\text{pre-HbAlc}] = X$ and $k_2 = k_b/\text{hemo}$, then $X = [G] - X - [A₀] = [A₀] - X$. Substituting yields:

$$dX/dt = k_1(([G] - X)([A₀] - X)) = X-k_1X/k_2.$$

Rearranging:

$$dX/dt = k_1((X^2 - ([G] + [A₀] + 1/hemo)X + [G]A₀)),$$

Applying the quadratic equation to find the roots of the expression within the parentheses: let $B = -([G] + [A₀] + 1/hemo)$ and $M = (B^2 - 4G[A₀])^{1/2}$. Then the roots are: $R_1 = -(B + M)/2$ and $R_2 = -(B - M)/2$. Now, $dX/dt = k_1(X - R_1)(X - R_2)$ and

$$dX/(X - R_1)(X - R_2) = 1/M \int dX = \ln (X/R_2) = \ln (X/R_1).$$

(2)

Then, $k_1$ is the slope of the plot of

$$\frac{1}{M} \ln \frac{(X - R_2)/(X - R_1)}{versus t}.$$

From the equilibrium concentrations of products and reactants the equilibrium constant may be calculated as:

$$k_{eq} = \frac{[\text{pre-HbAlc}]}{[G][\text{HbA}]},$$

and then $k_b = k_{eq}/hemo$.

(3, 4)

Rearrangement of Equation 2 provides an expression for calculating the concentration of pre-HbAlc at any time given the appropriate
rate constants and starting conditions:
\[
[\text{pre-HbA}_{1c}] = \frac{R_1 R_2 (\text{e}^{	ext{k}_{\text{r}}} - 1)}{(R_2 \cdot \text{e}^{	ext{k}_{\text{r}}} - k_1)}.
\]

The determination of \( k_0 \): Phase II—\( k_0 \) is the apparent first-order rate constant for the formation of stable HbA, via the Amadori rearrangement. Basic rate equation:
\[
d[A_{1c}] / dt = X_t - k_0
\]
then,
\[
k_0 = \frac{1}{X_t} (\Delta[A_{1c}] / \Delta t)
\]
where \( \Delta[A_{1c}] / \Delta t \) is the slope of the linear portion of the HbA, synthesis progress curve.

RESULTS
As an example of the data obtained in our experiments, Fig. 3A shows a set of progress curves for the formation of pre-HbA, at pH values ranging from 6.0 to 7.6. As the pH is decreased so is the equilibrium concentration of pre-HbA, from a maximum of 10.7% at pH 7.6 to 2.8% of the total hemoglobin at pH 6.0. The initial rate of formation appears to gradually increase with decreasing pH.

When the data from these experiments are plotted according to Equation 2, the linear plots shown in Fig. 3B are produced. The slope of each line is equal to \( k_0 \), the apparent second-order rate constant for the formation of pre-HbA, at each pH. It can be seen that \( k_0 \) increases from pH 7.6 to 6.3 and then decreases at pH 6.0.

Fig. 4 shows the dependence of \( k_0 \), \( k_{-1} \), and \( k_n \) on hydrogen ion concentration. In Fig. 4A, \( k_0 \) is seen to approximately double from pH 7.6 to 6.3. The rate of increase is a maximum between pH 7.0 and 6.7. Fig. 4B shows a gradual increase in \( k_{-1} \) as the pH is lowered from 7.6 to 7.0 and a more rapid, approximately linear, decrease below pH 7.0. The equilibrium constant for the formation of pre-HbA, is shown in Fig. 4C to vary in an approximately sigmoidal fashion with pH.

The effects of pH on the formation of pre-HbA, are complex, but provide insight into the mechanism of the glycosylation reaction. We have been able to interpret our results on the basis of a previously studied model of Schiff base formation. Jencks (14) has studied the effect of pH on the condensation of hydroxylamine with acetone by ultraviolet and infrared spectroscopies. This reaction results in Schiff base formation which is chemically analogous to the formation of pre-HbA,.

As the pH is lowered from 7.6 to 7.0, the equilibrium constant for the formation of pre-HbA, decreases so is the equilibrium concentration of pre-HbA,.

The decrease in the reaction rate as the pH was lowered further was attributed to the protonation of the hydroxylamine to form the conjugate acid which is unreactive. At low pH, under conditions of rapid dehydration and a decreased concentration of the reactive form of the hydroxylamine, the condensation of the amine and acetone becomes rate-limiting.

This analysis of the mechanism of acetone/hydroxylamine condensation was facilitated by the fact that the acid-catalyzed dehydration and the initial addition reaction were apparently rate-determining over separate pH ranges. This is not true in the case of pre-HbA, formation. As in the model system of Jencks (14), acid catalysis appears to occur over the same pH range.

In order to examine these two pH effects separately, we have been able to mathematically calculate the isolated effect of protonation on the apparent second-order rate constant \( k_0 \) and the apparent first-order rate constant \( k_{-1} \). Although the total concentration of the \( \alpha \)-amino group is a constant at all pH values, the reactive form decreases with decreasing pH as the group becomes protonated. Using the data of Garner et al. (16), we have estimated the concentration of the reactive unprotonated \( \alpha \)-amino group at pH values within our experimental range. Then Equation 5 was used to generate a series of plots such as shown in Fig. 3A.
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The effect of acid catalysis on the variations of the kinetic parameters with pH. The results of this analysis are compared with experimental data in Fig. 6.

The equilibrium constant for the reaction should not be affected by catalytic processes including that caused by the hydrogen ion. As is seen in Fig. 6A, the experimental results are very close to those predicted by the isolated effect of α-amino group protonation. Both curves closely approximate the titration curve of the α-amino group (16).

Theoretically, the rate constant for the dissociation reaction, should not be affected by the degree of protonation of the α-amino group. Our calculated results for $k_{-1}$ are shown in Fig. 6B and verify this expectation. The experimental data deviate significantly from this prediction, showing a linear 6-fold increase from pH 7.0 to 6.0. This effect is explained by the isolated effect of acid catalysis and is similar to the results of progress curves of pre-HbA1c formation based on the calculated concentration of reactive α-amino group present at each pH. The resultant data were then analyzed in the same manner as the actual experimental data to yield an estimate of the isolated effect of protonation of the amino group on the rate and equilibrium constants. This analysis factors out
of Jencks (14) whose model system also showed catalysis below pH 7.0 with an 8-fold increase in the reaction rate from pH 7.0 to 6.0.

The effect of pH on $k_{1}$ and $k_{\alpha}$ can be explained on the isolated bases of acid catalysis and amino group protonation, respectively. The effect of pH on $k_{1}$ is more complex. As seen in Fig. 6C, if protonation of the amino group was the only process affecting this rate constant, a gradual decrease in its value would be seen with decreasing pH. This is not the case. $k_{1}$ actually increases with falling pH over the range of 7.6 to 6.3. This result can be understood as the combined effect of acid catalysis and protonation. The contribution of each of these effects to the dependence of $k_{1}$ on pH may be estimated by assuming that the experimental results are an average of the two processes. Since both the experimental, or average, relationship and the predicted effect of protonation are known, a third curve may be drawn. This graph, which is shown in Fig. 6C, should be similar to that experimentally produced by $k_{1}$ because this parameter exhibits isolated acid catalysis. Comparison of Figs. 6, B and C, shows this to be true.

Once the equilibrium concentration of pre-HbA1c is achieved, further increases in total HbA1c are due to the formation of stable HbA1c via the Amadori rearrangement. Fig. 7A shows the progress curves for the relatively slow linear increase in total HbA1c as pre-HbA1c is rearranged to the stable ketoamine isomer. The progress curves for this reaction are linear, as predicted, over the entire range of pH values tested. When Equation 7 is used to analyze these data, the apparent first-order rate constant for the Amadori rearrangement may be calculated. Fig. 7B shows the plot of this rate constant versus pH. The rate of the reaction increases with decreasing pH in a linear fashion.

The effects of other molecules which physiologically interact with hemoglobin on $k_{1}$, $k_{-1}$, and $k_{\alpha}$ are shown in Fig. 8. The formation of pre-HbA1c under an atmosphere of air at pH 7.30 was chosen as the control condition as it approximates the physiologic conditions of pH and oxygen saturation within the red cell. When the reaction was performed under anaerobic conditions so that hemoglobin is deoxygenated, there is a significant increase in $k_{1}$ but not in $k_{-1}$. This results in a significant increase in $k_{\alpha}$.

In the presence of 5% CO₂, there is a significant and proportionate decrease in both $k_{1}$ and $k_{-1}$, resulting in an unaltered $k_{\alpha}$. The substitution of an atmosphere of 100% CO does not significantly alter any of the kinetic parameters.

The addition of 2,3-DPG to the reaction medium at the physiologic concentration of 4.2 mM has no statistically significant effect on either $k_{1}$ or $k_{-1}$ in the presence of normal atmosphere. The equilibrium constant is slightly but significantly increased under these conditions. The effect of 2,3-DPG under anaerobic conditions appears to be minimal, producing results similar to those obtained without DPG. The rate constants for the formation of the 1-glucose-hemoglobin adduct are essentially the same as for experiments with d-glucose.

**DISCUSSION**

We have presented a method which simplifies the determination of the kinetic parameters of HbA1c formation. The initial determination of these rate constants by Higgins and Bunn (9) monitored the glycosylation of hemoglobin with radiolabeled glucose. Application of this method required the purification of the [¹⁴C]glucose prior to use. It was also necessary to incubate both HbA₀ and HbA₁c with glucose in separate experiments so that the reactions involving sites of glycosylation other than the β chain α-aminogroup could be factored out of the subsequent analysis. This methodology also necessitated the chemical trapping of pre-HbA₁c with NaCNBH₃. These procedures have been eliminated by the use of cation-exchange HPLC to directly monitor HbA₁c formation so that $k_{1}$, $k_{-1}$, and, if desired, $k_{2}$ may be determined in a single experiment. This simplification has allowed us to extend the observations of Higgins and Bunn (9) to a wider range of experimental conditions, which for the first time include examination of the effects of hemoglobin ligands on the kinetics of HbA1c formation.

Mortenson and Christophersen (10) examined the kinetics of pre-HbA₁c formation under a limited set of conditions by isoelectric focusing. This method has the advantage of being

![Fig. 7](image-url)
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Fig. 8. The effects of hemoglobin ligands on the kinetic parameters of pre-HbA_{1c} formation. Control experiments were performed under air. All experiments were conducted with 110 mM glucose and 0.2 mM HbA at a pH of 7.3. Deoxy-Hb experiments were performed under 100% N₂. CO₂ experiments were under an atmosphere of 5% CO₂ in air. CO experiments were conducted under 100% CO. 2,3-DPG was used at the physiologic concentration of 4.2 mM. Data are the average of three experiments ± 1 S.D. p values were calculated with the two-tailed t test.

able to separate stable HbA_{1c} from pre-HbA_{1c}. The rate constants k₁ and k₁₋₋₁ were determined by estimating the initial slopes of the progress curves of pre-HbA_{1c} formation and dissociation. The mathematical analysis we have presented allows these kinetic constants to be calculated from linear plots of data from a single experiment.

The rate constants k₁ and k₁₋₋₁ are not simple kinetic constants dependent only on the chemistry of the condensation of glucose with the α-amino group of the hemoglobin β chain. These parameters are macroscopic rate constants reflecting the effect of any change in conditions which alters the rates of the reactions. Fig. 9 indicates that glucose, 2,3-DPG, CO₂, and the hydrogen ion directly interact with the α-amino group of the β chain and that oxygen binding to hemoglobin alters the position of this chemical group. Our simplified method of analysis has allowed us to examine the effects of each of these species on the glycosylation reaction.

Our results on the effect of pH on the formation of pre-HbA_{1c} conform to the mechanism derived by Jencks (14) for general Schiff base formation. Higgins and Bunn (9) determined the three rate constants for the formation of HbA_{1c} at pH 7.3. Our results are in close agreement with theirs at this pH. These investigators also examined the effect of pH on k₁. Their results show an approximately linear decrease in this parameter as pH is decreased from 8 to 5.5. This result differs from our experiments which show an increase in k₁ over this pH range. However, the results of Higgins and Bunn (9) are similar to our calculated effect of isolated α-amino group protonation (Fig. 6C). This disparity in results would be expected if the NaCNBH₃ used by Higgins and Bunn to chemically trap pre-HbA_{1c} was reactive not with pre-HbA_{1c}, but with the initial condensation product of glucose and the α-amino group (Fig. 5). In this model, the catalytic effect of the hydrogen ion on the dehydration step of the reaction would not be detected.

The decrease in k₁ at pH values below 6.3 may be due to a third effect of hydrogen ion concentration which potentially alters the equilibrium of the ring-chain tautomerism of glucose (see Fig. 9). A variety of chemical methods has been used to show that the acyclic or reducible form of glucose is present only as a very minor proportion of the total glucose (17). Los et al. (18) used polarographic methods under conditions close to those used in our experiments (pH 6.9, 0.1 M phosphate buffer) to show that only 0.0026% of glucose was present in the reactive form. By reasoning similar to that applied to amino group protonation, this implies that a small change in the equilibrium between the ring and chain forms of glucose could produce a large change in k₁ and k₋₋₁ but not in k₁₋₋₁.

Recent reviews (19, 20) indicate that only one study has examined the effect of pH on the ring-chain tautomerism of glucose and that the theoretical interpretation of the experimental data was in error (21). However, quantitative results from these experiments indicate a decrease in the reactive form of glucose as pH is decreased over the range studied in our experiments. Overend et al. (22) have studied the effects of pH on the ring-chain equilibrium of D-ribose and 2-deoxy-L-ribose. Both of these sugars showed decreased proportions of their acyclic forms as the pH was decreased over a range of 10 to 6 (22). By analogy to these experiments, the plateau and decrease in k₁ below pH 6.3 could be due to a decrease in
the reactive form of glucose. There is clearly no such effect on $k_{-1}$, and there may be an effect on $k_{eq}$. Instability of the hemoglobin molecule prevented extending these observations below pH 6.0.

That the aldimine form of HbA$_t$ undergoes the intermolecular Amadori rearrangement was proposed by Bunn et al. (3) with supporting evidence provided by Koenig et al. (4). This rearrangement has been shown in model systems to be acid-catalyzed and is thought to occur by the mechanism shown in Fig. 10 (23). As was shown in Fig. 7B, our results exhibit the expected catalytic effect over the entire range of experimental hydrogen ion concentrations examined.

Hemoglobin is a highly complex molecule that functionally interacts with several other molecular species, including oxygen, CO$_2$, CO, and 2,3-DPG. Hemoglobin binds oxygen at four sites which are distant from the $\beta$ chain amino terminus to which glucose adds to form HbA$_{1c}$. The binding of oxygen causes a series of conformational changes throughout the hemoglobin molecule which include an alteration in the position of the two $\alpha$-amino groups of the $\beta$ chains so that the distance between them increases from 16 to 20 Å (7). This change in conformation is critical to the functional interaction of 2,3-DPG with hemoglobin. This change in position could affect the rate of pre-HbA$_{1c}$ formation by altering the chemical environment of the $\alpha$-amino group or by changing the accessibility of this group to glucose binding. Our results show that deoxygenated hemoglobin has an increased $k_i$ and unchanged $k_{-1}$ resulting in a greater $k_{eq}$ when compared with oxygenated hemoglobin. These results help explain the data of Smith et al. (24) who showed in an erythrocyte tissue culture system that HbA$_{1c}$ was formed at a faster rate under anaerobic conditions. This result was apparently due, at least in part, to an increase in the equilibrium concentration of pre-HbA$_{1c}$.

Carbon monoxide binds to hemoglobin in a manner analogous to oxygen. One would therefore expect no significant change in the kinetics of pre-HbA$_{1c}$ formation when oxygen is replaced by CO. This expectation is confirmed by our results.

Garner et al. (16) have demonstrated that the p$K_a$ of the $\beta$ chain $\alpha$-amino group of deoxyhemoglobin is 6.8, which is less than the value of 7.0 reported for carbon monoxide hemoglobin. The lesser p$K_a$ would mean that a greater proportion of the $\alpha$-amino group would be in the deprotonated form at a given pH. This would potentially account for the experimental results of increasing $k_i$ without altering $k_{-1}$.

Hemoglobin is an important physiologic carrier of CO$_2$, a normal by-product of cellular metabolism. Kilmartin and Rossi-Bernardi (25) have shown that CO$_2$ is carried by the $\alpha$-amino groups of both the hemoglobin $\alpha$ and $\beta$ chains. One might therefore expect CO$_2$ to act as a blocking agent to the formation of pre-HbA$_{1c}$, much as protonation of this group does. Experimentally, this is not the case. In the presence of physiologic concentrations of CO$_2$ proportionate decreases in both $k_i$ and $k_{-1}$ are noted. This results in an unaltered $k_{eq}$. Carbon dioxide apparently chemically inhibits the actual condensation reaction of glucose and the amino group without affecting the equilibrium concentration of pre-HbA$_{1c}$.

Utilizing x-ray diffraction techniques, Arnone has shown that 2,3-DPG lies in the central cleft of the hemoglobin molecule along the 2-fold axis of symmetry (26). This highly anionic molecule forms ionic bonds with valine 1 and histidines 2 and 143 of both $\beta$ chains and with lysine 82 of either $\alpha$ chain. The bonds to the valines are via the $\alpha$-amino group. From this model, we would expect that the binding of glucose would be blocked by the presence of 2,3-DPG. This expectation is supported by the data of McDonald et al. (27) who showed that 2,3-DPG binds less avidly to HbA$_{1c}$ and has a diminished effect on oxygen affinity. Our experimental results differ from this conclusion showing insignificant changes in $k_i$ and $k_{-1}$ and a small, but significant, increase in $k_{eq}$ implying an increased equilibrium concentration of pre-HbA$_{1c}$. Under anaerobic conditions where 2,3-DPG has a much greater affinity for its binding site (28), no significant changes are seen in comparison to deoxyhemoglobin experiments without 2,3-DPG. A possible explanation for our inability to demonstrate a 2,3-DPG effect is that phosphate from the buffer system occupied the molecule’s binding site.

The increase in the $k_{eq}$ in the presence of 2,3-DPG is in agreement with the work of Smith et al. (24) who examined the effect of 2,3-DPG on HbA$_{1c}$ formation in red cells and found an increase in the rate of formation. This result can now be interpreted as the result of an increased concentration of pre-HbA$_{1c}$ in the presence of 2,3-DPG.

The formation of pre-HbA$_{1c}$ from L-glucose has the same kinetic parameters as when D-glucose is used, implying that the site of the reaction between glucose and the $\alpha$-amino group is not sterically defined.

The glycosylation of HbA$_{1c}$ has been studied as the prototype of the nonenzymatic glycosylation of proteins. Understanding this process has led to speculation that the numerous, and nearly inevitable, complications of diabetes are a result of nonenzymatic glycosylation of specific proteins. This has led to new avenues of investigation with the eventual goals of prevention and treatment of these conditions (29). Our work has provided insight into the mechanism of nonenzymatic glycosylation and has defined the effects of several hemoglobin ligands on the kinetics of HbA$_{1c}$ formation.

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