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Accelerated autoxidation and heme loss due to instability of sickle hemoglobin

(erythrocyte/membrane/superoxide)

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ABSTRACT **The pleiotropic effect of the sickle gene suggests that factors in addition to polymerization of the mutant gene product might be involved in sickle disease pathobiology. We have examined rates of heme transfer to hemopexin from hemoglobin in dilute aqueous solution (0.5 mg of Hb per ml) at 37°C. HbO_2 S loses heme 1.7 times faster than HbO_2 A, with apparent rate constants of 0.024 hr^{-1} and 0.014 hr^{-1}, respectively. In contrast, Hb A and Hb S behave identically in their MetHb forms (very rapid heme loss) and their HbCO forms (zero heme loss). This indicates that the faster heme loss from HbO_2 S is due to accelerated autoxidation (HbO_2 → MetHb) rather than to some other type of instability inherent in the relationship of sickle heme to its pocket in globin. This interpretation is supported by spectrophotometric measurement of initial rates of MetHb formation during incubation at 37°C. This directly shows 1.7 times faster autoxidation, with apparent rate constants of 0.050 hr^{-1} for HbO_2 S and 0.029 hr^{-1} for HbO_2 A. While the participation of this process in the cellular pathobiology of sickle erythrocytes remains unproven, the present data are consistent with, and perhaps help explain, two prior observations: the excessive spontaneous generation of superoxide by sickle erythrocytes; and the abnormal deposition of heme and heme proteins on membranes of sickle erythrocytes.**

The pathophysiology of sickle cell anemia is explained ultimately by the presence of sickle hemoglobin (Hb S), a mutant gene product well known for its tendency to polymerize at low oxygen tension. However, the sickle gene has remarkably pleiotropic effects, most evident in the multitude of membrane defects characteristic of sickle erythrocytes (1, 2). Some of these membrane lesions are implicated in disease pathophysiology, but the mechanisms linking presence of the mutant gene product with development of membrane defects remain obscure. As a potential explanation, we have hypothesized that sickle cell disease is partly a disorder of autoxidation and iron decompartmentalization (2). This concept emphasizes the importance of two components in cellular pathobiology of sickle erythrocytes: their excessive spontaneous generation of superoxide (3) and their abnormal amounts of membrane-associated heme iron (much of which is believed to be in the form of hemichromes, low-spin ferric denatured hemoglobins) (4, 5, 32).

Theoretically, both of these findings could reflect an instability of Hb S. In fact, Hb S does tend to precipitate and form hemichromes during vigorous mechanical agitation (6), but the physiologic analogue of this mechanical instability has not been identified. Furthermore, it has not yet been demonstrated that the excessive superoxide generation by sickle erythrocytes (3) actually reflects an abnormal molecular behavior of Hb S, rather than a simple difference in levels or efficacy of cellular antioxidants. Thus, neither the excess membrane-associated heme iron nor the excess superoxide generation have been adequately explained by existing data.

Consequently, we have begun to examine molecular behaviors of Hb S other than its well-known polymerization tendency. In the present report, we compare rates of heme transfer from HbO_2 S and A to the heme-binding glycoprotein hemopexin (Hpx). The results demonstrate an inherent instability of Hb S and explain this on the basis of accelerated autoxidation of the heme moiety in Hb S. While a specific role for this abnormality in cellular pathophysiology remains to be proven, some prior observations potentially can be explained by these data.

METHODS

Materials. Chromatography matrices were obtained from Pharmacia, and all other reagents were obtained from Sigma.

Hpx and Haptoglobin (Hap). As described (7, 8), human phenotype 1-1 Hap was isolated from plasma by affinity chromatography using turkey cyanMetHb immobilized on Sepharose CL-4B. Rabbit Hpx was isolated from serum using $HClO_4$ precipitation, ion-exchange chromatography over DEAE-Sepharose CL-6B, and affinity chromatography on wheat germ lectin-Sepharose 6MB, as described (9–11). Both reagents were >95% pure by polyacrylamide gel electrophoresis.

Hb Preparations. Fresh whole blood was drawn from volunteer nonsmoking donors. One individual with sickle-cell trait (Hb AS) provided both Hb A and Hb S. Additional Hb A samples were obtained from two normal (Hb AA) donors, and additional Hb S samples were obtained from three donors with sickle-cell anemia (Hb SS).

Hb was isolated from erythrocyte lysate by ion-exchange chromatography on DEAE-Sepharose CL-6B. Eluates were assessed for purity by using isoelectric focusing, dialyzed against the assay buffer (see below), concentrated by Amicon filtration, and passed through a Sephadex G100 column. Other preparative steps recommended by Caughey and Watkins (12) were not used because it was deemed essential to minimize both elapsed time and exposure of Hb to chromatographic procedures. However, in one case, samples of Hb A and Hb S were prepared in parallel and actually dialyzed against each other in the above steps. All preparative steps were performed at 4°C, and buffers used for Hb preparation were made with distilled deionized water treated with deferoxamine immobilized on Sepharose CL-4B to remove traces of iron. When necessary, Hb preparations were added dropwise to liquid nitrogen for short-term storage at −70°C.

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Abbreviations: Hap, haptoglobin; Hpx, hemopexin.

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At the time of use, amount of contaminating MetHb was calculated as described by Winterbourn after measurement of absorbance at 560, 577, 630, and 700 nm (13). Total Hb concentration was determined after conversion to cyanMet-Hb. For experiments reported here, only samples having <2% MetHb were used. Intentional conversion of HbO_2 to MetHb (≥90%) was achieved by incubation of the former with $K_3Fe(CN)_6$ in 1.5-fold excess over heme, followed by passage through a Sephadex G-25 column. HbCO was prepared by fully equilibrating HbO_2 with 100% CO.

Heme Transfer Assay. Purified Hb, Hap, and Hpx were used in 0.1 M sodium phosphate buffer (pH 7.2). Hpx and Hb were admixed to final concentrations of 0.5 mg of Hb per ml and 2.0 mg of Hpx per ml, which provides 1 mol of Hpx per mol of heme. After bringing the mixture to 37.0°C (±0.1°C), 250-μl aliquots were removed at various time points and added to 167 μl of Hap (3 mg/ml) to terminate any further heme loss (14). For MetHb, the zero-time aliquot was obtained immediately; for HbO_2 and HbCO the zero-time aliquot was obtained after 30 min (so the small contribution from any preexisting MetHb would be included in the zero-time sample). After 15 min, the Hap/Hb/Hpx mixture was applied to a Sephacryl S-300 column (1.5 × 107 cm) run at 10 ml/hr to physically separate newly created Hpx/heme from residual globin/heme (now running as Hb/Hap complex). One milliliter fractions were screened for heme content (Soret absorption), an example of which is depicted in Fig. 1. Fractions comprising the Hap/Hb and the Hpx/heme peaks were separately pooled. Heme content of the peaks was determined both by measuring absorbance at 398 nm after solubilization in concentrated formic acid and by the benzidine method (15); comparable results were obtained with the two techniques. After subtraction of any heme in the zero-time Hpx peak, the appearance of heme in the Hpx peak as a function of time was calculated. The time over which measurements were made varied depending on Hb type: 24 hr for HbO_2, 36 hr for HbCO, and 75 min for MetHb.

Several types of control experiments were done to validate this method (data not shown). When the experiment shown in Fig. 1 was repeated using MetHb labeled with ^{14}C (by prior carbamoylation of globin), all radioactivity partitioned into the Hap/Hb peak and none partitioned into the Hpx peak. Likewise, if Hpx is eliminated and Hap and Hb are admixed in the ratios described above, neither protein nor heme is found in fractions to the right of the Hap/Hb peak. Thus, there is no contamination of the Hpx peak with Hb. In a separate study, the experiment shown in Fig. 1 was repeated so that we could compare the effect of admixing Hpx with Hb at the start of the incubation (our standard protocol as described above) with the effect of admixing the Hpx only after the end of the Hb incubation. As expected (see *Discussion*), no heme was taken up by the Hpx added at the end of the incubation, indicating that negligible heme loss from globin is detectable in the absence of an alternative heme-binding substance.

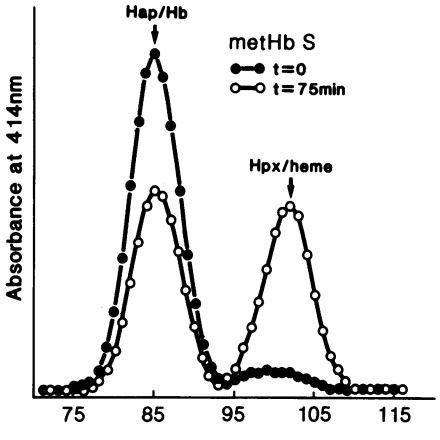

FIG. 1. Example of heme transfer from Hb to Hpx. Hb and Hpx were admixed and incubated at 37.0°C. At various time points, aliquots were added to excess Hap to stop the reaction. Application to a Sephacryl S-300 column physically separated residual globin–heme (now traveling as Hap/Hb) from Hpx–heme appearing due to heme loss from globin, as shown here for column fractions screened for Soret absorbance.

MetHb Assay. Samples of HbO_2 A and S were also incubated (without Hpx) at 1 mg/ml in 0.1 M sodium phosphate buffer (pH 7.2) at 37°C (±0.1°C) in a Beckman DU8 Spectrophotometer. Every 10 min we measured absorbance at 560, 577, 630, and 700 nm, and the amount of MetHb was calculated as described (13).

RESULTS

After admixture of Hb and Hpx, heme transfers to the Hpx if it first dissociates from globin (16). Our comparison of HbO_2 A and HbO_2 S is shown in Fig. 2, which plots the fraction of heme remaining associated with globin as a function of time. Thus, heme loss is evident in the downward slope of the data points, and the apparent rate constants derived from these data are presented in Table 1. It is evident that HbO_2 S loses heme ≈1.7 times faster than HbO_2 A loses heme.

In contrast, HbCO forms of Hb A and Hb S are indistinguishable and have no detectable heme loss (Fig. 2). HbCO forms actually were evaluated over 36 hr and showed no heme loss even in that time period. The estimated degree of error for any data point is such that HbCO theoretically could have lost as much as 2% of its heme in 36 hr without being detected. Clearly, however, this amount of loss would be negligible compared to heme loss from HbO_2 or MetHb. So for purposes of analysis we will consider rate of heme loss from either HbCO to be zero. This, in fact, is consistent with previous data on HbCO A and HbCO F analyzed by a different technique (14).

Similarly, Hb S and Hb A are identical when compared in their MetHb forms, although here the heme loss is extremely rapid (Fig. 2). As expected (14), heme loss from MetHb is nonlinear, so we have used the first time point to estimate an apparent rate constant for comparative purposes (Table 1).

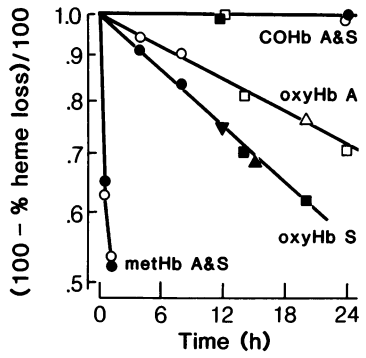

FIG. 2. Loss of heme from Hb A and Hb S in O_2, Met, and CO forms. Heme loss was determined as illustrated in Fig. 1 and as described in *Methods*. Vertical axis plots the fraction of globin retaining its heme [(100 − % heme loss)/100]. One Hb AS donor provided both Hb A (□) and Hb S (■). Other Hb A data are derived from two Hb AA donors (○, △), and other Hb S data are derived from three Hb SS donors (●, ▼, ▲). One pair of Hb A and Hb S samples (○, ●) were prepared in parallel and were actually dialyzed against each other during preparative steps.

Table 1. Apparent rate constants for data in Figs. 2 and 3

	k, hr^{-1}	
	Heme transfer from Hb to Hpx	MetHb formation
HbO_2 A	0.014	0.029
HbO_2 S	0.024	0.050
HbCO A and S	0	
MetHb A and S	0.923*	

All Hb were examined in 0.1 mM sodium phosphate (pH 7.2). For heme transfer experiments, Hb concentration was 0.5 mg/ml, and temperature was 37.0°C (±0.1°C). For the MetHb formation experiment, Hb concentration was 1.0 mg/ml, and temperature was 36.7°C (±0.1°C). Values for k were calculated from slopes of the curves shown in Figs. 2 and 3.
*Determined for first time point only (+0.5 hr).

Our results are consistent with a previous report documenting a $T_{1/2}$ of ≈100 min for heme loss from MetHb A (14).

Since these data are interpreted to indicate an accelerated rate of autoxidation for HbO_2 S (as discussed below), we directly examined autoxidation during aerobic incubation. As shown in Fig. 3, HbO_2 S converts to MetHb at a faster rate than HbO_2 A, and apparent rate constants (Table 1) again show a 1.7-fold difference between HbO_2 S and HbO_2 A.

The data shown in Fig. 3 and some of that shown in Fig. 2 (square symbols) were obtained from the Hb AS donor. Thus, Hb A and Hb S used for comparison in some of these experiments had been subjected to the same intracellular environment prior to purification. Our experiments necessarily were done using very low Hb concentrations. However, we were able to perform a single heme loss experiment using 10-fold higher HbO_2 S concentration (5.0 mg/ml), and this yielded nearly identical results for heme loss to Hpx, with an apparent rate constant of 0.023 hr^{-1}.

DISCUSSION

As reflected in rates of transfer of heme from globin to Hpx (Fig. 2; Table 1), HbO_2 S loses heme slowly but 1.7 times faster than HbO_2 A loses heme. In contrast, heme loss from either MetHb is extremely rapid, but MetHb A and MetHb S behave identically in this regard. Likewise, HbCO forms of Hb A and Hb S are identical, but it is particularly important to note that heme loss is not even detectable from the HbCO forms. From these data, we conclude that the accelerated heme loss from HbO_2 S reflects a faster rate of Hb autoxidation, as opposed to some other kind of instability inherent in the relationship of sickle heme to its pocket in globin. Thus, Hb S is unlike the highly unstable Hb Koln, which is reported to lose ferric heme even more rapidly than Hb A loses ferric heme (17).

Data Interpretation. That our conclusion is justified is evident if we consider Eqs. **1–6** (in which G is globin; H is heme; GH is hemoglobin; Hpx is hemopexin; H^{2+} is ferrous heme; H^{3+} is ferric heme; and O_2^- is superoxide).

$$GH + Hpx \rightleftharpoons G + HpxH \quad [1]$$

$$GH \rightleftharpoons G + H \quad [2]$$

$$H + Hpx \rightleftharpoons HpxH \quad [3]$$

$$GH^{2+} \rightleftharpoons G + H^{2+} \quad [4]$$

$$GH^{3+} \rightleftharpoons G + H^{3+} \quad [5]$$

$$GH^{2+} + O_2 \longrightarrow GH^{3+} + O_2^- \quad [6]$$

Data on HbO_2 in Fig. 2 reflect the overall process of Eq. **1**, which in turn reflects two reactions, Eqs. **2** and **3**. However, Eq. **2** could take either of two forms, Eq. **4** or Eq. **5**. Since HbCO cannot autoxidize, our results for HbCO (Fig. 2) appear to establish that there is no heme loss in the absence of Hb autoxidation. Hence, the direct loss of ferrous heme from Hb (Eq. **4**) does not occur, and Eq. **5** is the accurate form of Eq. **2**. Consequently, Eq. **1** reflects Eq. **3** plus Eq. **5**, both proceeding to the right as written.

The affinity constant for association of heme and Hpx (Eq. **3**) is extremely high [1.9×10^{14} M^{-1} (16)], as is the affinity constant for association of heme and globin [$>10^{13}$ M^{-1} (16, 18)]. These affinity constants are so high that it is reasonable to assume that in our experiment there is no free heme unassociated with carrier protein. The affinities of globin and Hpx for heme are sufficiently similar that we cannot assume Eq. **1** proceeds only to the right. Nevertheless, under the

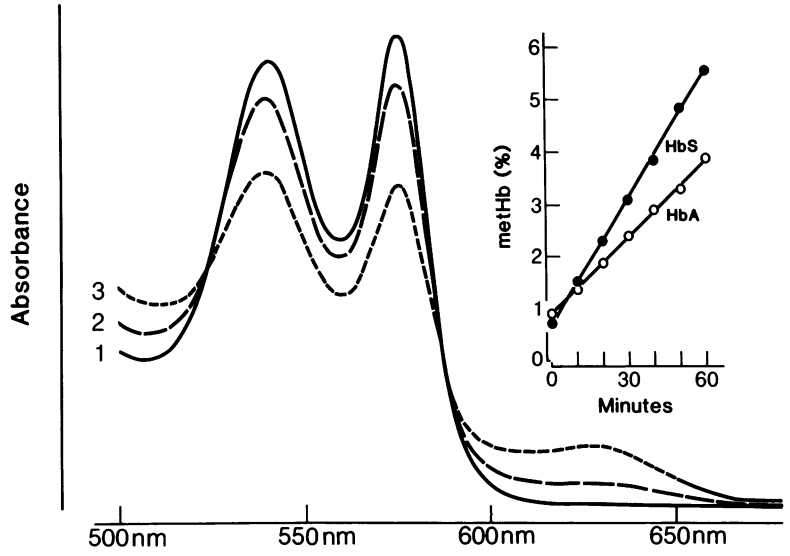

FIG. 3. Autoxidation of HbO_2 A and HbO_2 S. Example of absorption spectrum is shown for HbO_2 S incubated at 36.7°C: curve 1, zero time, curve 2, +6.5 hr; curve 3, +22.5 hr. MetHb formation is evident in diminishing absorption at 560 nm and 577 nm and increasing absorption at 630 nm. Absorption at 700 nm was noted to be rising above baseline by +18 hr (data not shown). (*Inset*) Percentage MetHb as function of time for preparations of HbO_2 A and HbO_2 S from the Hb AS donor used for data in Fig. 2.

conditions of our experiment, it is clear that Eq. 1 will be limited by dissociation of heme from globin in MetHb (Eq. 5).

However, Fig. 2 illustrates that heme loss from HbO_2 is very slow compared to heme loss from MetHb. Therefore, our HbO_2 results must reflect Eq. 6 (heme autoxidation) as the rate-limiting process. Consequently, we also measured MetHb formation spectrophotometrically during aerobic incubation at 37°C. The results (Fig. 3) directly demonstrate the accelerated autoxidation of HbO_2 S. Consistent with the above interpretations, the magnitude of difference between Hb S and Hb A (1.7-fold) is the same for measurement of MetHb formation as for heme loss *per se* (Table 1). That absolute apparent rate constants are lower for the heme transfer experiment presumably reflects the similar affinities of globin and Hpx for heme. Indeed, the equilibrium constant for Eq. 1 is reported to be 1 for loss of the first heme from a MetHb tetramer (16).

In reality, however, even Eq. 6 is oversimplified. Autoxidation of Hb is itself complicated, potentially involving not only interaction between heme and water (or OH^-), but also interaction between heme and other oxidants (e.g., peroxide and hydroxyl radical) derived from superoxide (19, 20). Moreover, Eq. 6 is written here without recognition of the fact that Hb is tetrameric, and all hemes in this tetramer are not equivalent. For example, α chains autoxidize perhaps 10 times more rapidly than β chains (21, 22), but ferric β chains lose heme $\approx$8 times more rapidly than ferric α chains lose heme (14). Furthermore, heme loss from the second ferric β chain is expected to be faster than heme loss from the first ferric β chain (16). Also, heme loss data would be expected to reflect increasing instability of globin as heme loss continues.

Indeed, it was for these reasons that we confined our measurements to the very initial period of MetHb formation (Fig. 3), which emphasizes the fast, truly autoxidative component of MetHb formation (19, 20). Similarly, our examination of heme transfer rates (Fig. 2) was limited to the first 25% of heme loss, representing an average loss of the first of the four hemes in Hb. By confining our studies to loss of the first heme, we have collected data that emphasize the behavior of β chains (16). It may or may not be the case that the α chains in Hb A and Hb S are behaving identically in our experiments. Thus, linearity of our HbO_2 data (Fig. 2) would appear to be a convenient accident, where these various processes balance each other. Nevertheless, the data in Figs. 2 and 3 must reflect the only difference in the sickle tetramer, its mutant β-chain composition. Consequently, the slopes of the HbO_2 curves as calculated in Table 1 directly reflect this and identify an abnormal molecular behavior of Hb S.

In this analysis, we have made no provision for the possibility that behavior of Hb dimer might differ from that of tetramer. However, it is unlikely that our data are relevant only to enhanced dimerization at low Hb concentration. In the first place, we found no significant effect of Hb concentration on apparent rate constant for heme loss to Hpx. In addition, although the proportion of dimer is higher in dilute Hb solution, the absolute concentration of dimer is greater at physiologic Hb concentration.¶ Consequently, we believe that our findings are relevant to events in the intact cell.

Implications for Cellular Pathobiology. Thus, the present data demonstrate a rate of autoxidation that is 1.7 times faster for HbO_2 S than for HbO_2 A. We note that an effect of this magnitude has also been preliminarily reported by Watkins *et al.* (24) as well as by ourselves (25). This is acceptably close to the average 2-fold excess of spontaneous superoxide formation by intact sickle erythrocytes that we earlier reported (3). Since Hb autoxidation is a relentless source of superoxide (Eq. 6), the present data provide support for our belief that those earlier results do reflect an abnormal molecular behavior of Hb S. Beyond this, however, it would be hazardous to assume that we can now draw firm conclusions regarding cellular pathophysiology.

For example, accelerated autoxidation of Hb S undoubtedly is modulated by numerous factors in the intact cell. The presence of cellular antioxidants might inhibit MetHb formations as reported for superoxide dismutase (19, 26). On the other hand, sickle erythrocytes are reported to be abnormally laden with both iron (27) and copper (28), both of which could accelerate MetHb formation. Regarding heme loss *per se*, its occurrence due to Hb autoxidation *in situ* would be diminished by enzymatic reduction of MetHb (which has not been reported to be abnormal in sickle patients). On the other hand, heme loss theoretically could be accelerated by effects of twice-normal amounts of superoxide-derived oxidants (3) upon Hb (29). Obviously, additional studies will be required to evaluate the enormous variety in which autoxidation and heme loss might be modulated in the context of the intact cell. It is likely that some of these factors account for the fact that the amount of superoxide generated by sickle erythrocytes from different patients is somewhat variable (3).

Aside from the potential harmful effect of accelerated oxidant generation upon the cell membrane [as reviewed elsewhere (2)], these findings potentially are related to the abnormal deposits of denatured hemoglobin associated with sickle erythrocyte membranes (4, 5), since MetHb formation is believed to be a necessary first step in ultimate formation of hemichromes (30). Although steady-state amounts of MetHb do not appear to be elevated in sickle cell cytosol, the accelerated MetHb turnover presumably would increase the number of Hb molecules at risk per unit time. Nevertheless, it is likely that accelerated MetHb formation is not itself a sufficient condition for hemichrome deposition. Thus, our understanding of specific steps leading to hemichrome deposition on sickle cell membranes still is incomplete.

It remains to be seen whether our use of Hpx as a surrogate for potential cellular heme-binding substances provides an accurate reflection of other effects of accelerated autoxidation on sickle cell pathobiology. As noted in *Methods*, the actual loss of heme from MetHb was detectable in the present experiments only because we admixed Hb with an alternative heme binding substance (Hpx). Conversely, however, these data suggest the possibility that one consequence of accelerated autoxidation of Hb S would be the transfer of heme from globin to other potential binders. Thus, these data could help explain the abnormal amounts of free heme found in sickle erythrocyte cytosol (31) and membranes (5, 32). However, many of the considerations noted in the preceding paragraph are also pertinent to this possibility, so our understanding of this process is incomplete as well. Nevertheless, the present data indicate that accelerated heme loss is a potential consequence of the sickle mutation.

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¶For example, for our standard conditions (0.5 mg of Hb per ml and 37.0°C) we calculate 17.4% dimer compared to 0.008% at Hb concentration of 30 g/dl; yet, the comparable absolute dimer concentrations are 2.7×10^{-6} M for the dilute solution and 71.3×10^{-6} M for the physiologic Hb concentration. This calculation assumes that the tetramer–dimer equilibrium can be expressed by $D^2/T = K$, where $T = H/4 - D/2$, H = [heme], D = [dimer], T = [tetramer], and $K = 1.1 \times 10^{-6}$ M at 37.0°C. This value for K is calculated from a value of 1.5×10^{-6} M at 21.5°C assuming a ΔH of −3900 cal (1 cal = 4.18 J) (23).

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