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Ligand kinetics of hemoglobin S containing erythrocytes

(sickling/rate of oxygenation/polymerization)

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ABSTRACT **Oxygen uptake of fully deoxygenated sickle (SS) erythrocytes is slower than that of normal (AA) erythrocytes, as demonstrated by the half-times of the overall oxygenation reactions: at 25° in an isotonic phosphate buffer the normal red cells have a $t_{1/2} = 82 \pm 4.7$ msec, as compared to sickle red cells where $t_{1/2} = 135 \pm 17.6$ msec. The effects of temperature, extracellular osmolality, and the presence of an antisickling agent (*n*-butylurea) on the rate of red cell oxygenation strongly suggest that the differences in oxygenation rates encountered with sickle red cells is directly related to the intracellular polymerization of deoxyhemoglobin S.**

Erythrocytes from persons with sickle cell anemia have an abnormal oxygen equilibrium curve, reflecting an increase in the partial pressure of oxygen needed to half-saturate the available binding sites (1–3). The mechanism of this shift is not clearly established, but it is connected with the intraerythrocytic polymerization of the deoxy form of hemoglobin (Hb) S (4, 5).

Rates of oxygen and carbon monoxide uptake by red blood cells were first measured 50 years ago by Hartridge and Roughton (6, 7), using the reversion spectroscope and their continuous flow, rapid reaction technique. Improvements in the detections system by Legge and Roughton (8) and by Forster *et al.* (9) were followed by contributions from the laboratories of Gibson (10), Roughton (11), and Forster (9). Subsequently, Sirs and Roughton (12) constructed an apparatus for the study of red blood cells, combining the split-beam detection system (9) with the stopped-flow principle. This apparatus or modifications of it have been used by later workers.

The findings of Hartridge, Roughton, Gibson, Forster and their colleagues have been summarized by Roughton (13, 14) and by Forster (15). The pure chemical kinetics of the reactions of Hb in solution and in the red cell appear to be the same. Nonetheless, the rates of reaction of Hb in the red cell are slower than in solution. It is noted that the discrepancy between the rate of a given process in Hb solutions and in the red cell suspension increases *pari passu* with the speed of the reaction in solution. The rate of the fast process, combination of Hb with oxygen, is 30-fold slower in the red cell at 37° than in solution.

Since the transit time for the erythrocytes in the pulmonary capillaries is about 700–800 msec, several questions arise regarding oxygen exchange in the Hb S-containing red cells that could have physiological significance. (*i*) How does the rate of oxygenation of sickled cells differ from normal cells? (*ii*) What is the relative contribution of the abnormalities of the red cell membrane and the polymerization of deoxy Hb S to the uptake or egress of oxygen in these cells? Recently, Messer *et al.* (16), using a continuous flow apparatus, reported that deoxygenated sickled cells had oxygenation rates different from those of normal (AA) cells.

To approach these questions we have utilized a custom-made stopped-flow apparatus specially designed for the study of red cell ligand kinetics. Our investigations explored the effects of variations in temperature and in extracellular osmolality, and the effects of an antisickling agent (*n*-butylurea) on the rate of red cell oxygenation. In addition, a high-density fraction of sickle red cells rich in irreversibly sickled forms (ISC), which possess rigid membranes (17), were compared to the low density fraction to help assess the effect of the abnormal membranes on ligand kinetics.

MATERIALS AND METHODS

Heparinized venous blood was obtained from patients homozygous (SS) and heterozygous (SA) for Hb S, as well as from normal individuals. All donors were followed in the Heredity Clinic associated with our laboratory, where our hemoglobin genotypes had been documented by clinical and laboratory studies. Hb F, as measured by the alkali resistance method (18), was less than 6.0% in all SS patients studied. All experiments were performed within 24 hr of blood donation.

The red cells were washed three times in an isotonic buffer, 0.15 M potassium phosphate, pH 7.35, and the packed cells were diluted 1 to 800 in the appropriate buffer. For certain experiments the osmolality of the suspending buffer was raised to about 430 mOsm per kg of H_2O by the addition of NaCl. Buffer osmolalities were measured with a Fiske Osmometer (model 330D).

The oxygenation reaction was studied by first deoxygenating the suspension of red cells in a tonometer by alternating vacuum and helium flushing. Red cell structure was examined with a Zeiss microscope equipped with differential interference contrast optics. After deoxygenation, a portion of each SS cell suspension used was fixed in formalin and found to contain 90–95% of the cells sickled or deformed. (A cell was classified as sickled when at least one pointy protuberance was observed on its surface and as deformed when its shape was distinctly different from a biconcave disc.) The deoxygenated red cell suspension was placed in one of the syringes of the stopped-flow apparatus. The other syringe contained 0.15 M potassium phosphate buffer, pH 7.35, which had been equilibrated with air at 24° or 37°.

The deoxygenation reaction of red cells was studied in an air-equilibrated red cell suspension (in 0.1 M potassium phosphate, 0.021 M NaCl buffer, pH 7.35) in one syringe and a solution of sodium dithionite (6 g/liter in deaerated 0.1 M potassium phosphate, adjusted to 300 mOsm per kg of H_2O with NaCl) in the other syringe.

For studies of the effect of butylurea on the rate of red cell oxygenation, 0.1 M butylurea was added to the buffers in which

Abbreviations: Hb, hemoglobin; AA, SS, and SA erythrocytes, normal, fully deoxygenated sickle, and sickle trait erythrocytes, respectively; ISC, irreversibly sickled cells.

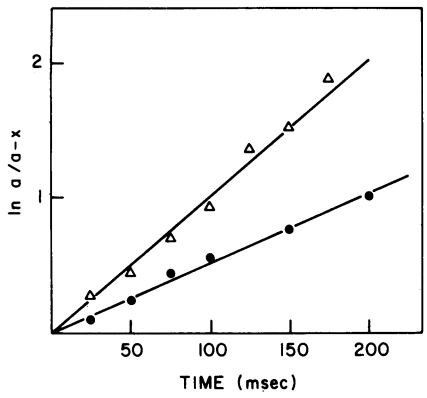

FIG. 1. Pseudo-first order plots for the kinetics of oxygenation of normal (△) and sickle (●) red cells suspended in 0.15 M potassium phosphate, pH 7.35. Temperature, 25° ± 1°; oxygen concentration (before mixing); 0.21 mM.

red cells were incubated and deoxygenated as well as to the air-equilibrated buffer used for oxygenation.

High- and low-density fractions of SS red cells were prepared essentially by the method of Murphy (19). Heparinized blood was centrifuged at 2000 × *g* at 4° for 15 min; after the plasma and buffy coat were removed, the red cells were transferred to a 16 × 100 mm polypropylene tube and centrifuged for 1 hr at 30°, at 15,000 rpm in a Sorvall RC-2B centrifuge using a SS-34 fixed-angle rotor. The fractions used for experiments comprised approximately the top 8–10% (light fraction) and the bottom 8–10% (high density, ISC-rich fraction) of the centrifuged cells.

Rates of red cell oxygenation and deoxygenation were determined with a Sirs-Roughton type stopped-flow apparatus (12) modified by one of us (J.B.W.) to include "state-of-the-art" mechanical, electrical, and optical components. The essential new feature of the detection system is the use of a fiber optic beam divider (American Optical Co.) to split the light beam emerging from the observation tube. The two half beams pass through interference filters (560 and 579 nm) to paired photomultipliers. The sum of the amplified signal outputs is proportional to the sum of the absorption differences at 560 and 579 nm. By use of a transient recorder (Biomation model 802), the kinetic events could be plotted with an X-Y recorder (Hewlett Packard model 7004B) so that half-times ($t_{1/2}$, msec) of the oxygenation or deoxygenation reactions could be determined directly. This system minimizes artifacts caused by settling or unequal distribution of red cells in the observation chamber, or by other sources of change in light scattering occurring during the course of the reaction, and thereby extends the useful range of measurement even of slow reactions (e.g., replacement of Hb-bound oxygen by carbon monoxide) to include the full course of the reaction.

Aliquots from the two syringes, one containing the red cell suspension, the second containing the oxygenation or deoxygenation buffer, were driven simultaneously into a mixing chamber, of eight 1-mm diameter tangential jets (12, 20) leading to a 2-mm diameter, 2-cm long observation tube. The apparatus was contained in a thermally regulated water bath.

The oxygenating buffer was air-equilibrated at 37°, so that the concentration of dissolved oxygen was 0.21 mM; since the buffer was kept in a closed system as the temperature was lowered, a constant amount of oxygen was maintained.

Based upon five to seven determinations for each blood sample studied, the half-time values ($t_{1/2}$, msec) varied from the mean value by no more than ±10%.

RESULTS

The rates of oxygenation of AA, SS, and SA red cells follow pseudo-first order kinetics (Fig. 1), and plots of the progress of the reaction are linear to more than 70% completion. The rates of oxygenation were expressed as the half-times of the oxygenation reactions ($t_{1/2}$, msec), as shown in Table 1. At 25° in isotonic buffer, there was a clear difference between the oxygenation rates of normal (AA) red cells ($t_{1/2}$ = 82 ± 4.7 msec) and of SS red cells ($t_{1/2}$ = 135 ± 17.6 msec). These differences were also evident at 37°, where $t_{1/2}$ (AA) = 70 ± 4.8 msec and $t_{1/2}$ (SS) = 105 ± 9.5 msec. The oxygenation rates of sickle trait (SA) red cells ($t_{1/2}$ = 83 ± 5.3 msec at 25° and 69 ± 8.9 msec at 37°) were quite similar to those of normal (AA) red cells in isotonic buffer.

In order to determine the role of intracellular polymerization of deoxy Hb S, SA red cells were suspended in a hypertonic buffer (430 mOsm per kg of H_2O) so as to induce osmotic shrinkage and thereby raise the intracellular Hb concentration; under conditions similar to these, SA cells were found to sickle to the same extent as SS cells at each level of deoxygenation (21). As seen in Table 1, the oxygenation rate of these SA cells in the hypertonic buffer was indistinguishable from that of SS red cells suspended in isotonic buffer (300 mOsm per kg of H_2O). Thus, osmotic shrinkage resulted in an increase in the $t_{1/2}$ of SA cells ($t_{1/2}$ = 128 ± 25.3 msec at 25° and 105 ± 28.6 msec at 37°) compared to the same cells in isotonic buffer. Suspension in hypertonic buffer did not produce an increase in the $t_{1/2}$ of SS red cells, but a slight increase in the $t_{1/2}$ of the oxygenation reaction was seen with normal red cells.

In order to elucidate the role of the cell membrane in these kinetic events, we compared the oxygenation rates of a high-density fraction of SS red cells in which the proportion of irreversibly sickled cells was increased (52–60% ISC), with a low-density fraction containing 3–20% ISC. The high-density fraction has a much higher mean corpuscular Hb concentration and the ISC have distorted membranes whose morphological abnormalities persist after oxygenation. The results of these experiments, shown in Table 2, indicate that the oxygenation rates of the low-density, low ISC top fraction of SS red cells ($t_{1/2}$ = 107 ± 0.7 msec at 37°) did not differ significantly from the high-density, bottom fraction containing ISC ($t_{1/2}$ = 112 ± 14.1

Table 1. Rates of oxygenation ($t_{½}$ msec ± SD) for Hb AA, Hb SS, and Hb SA red cells, pH 7.35

Red cells	300 mOsm		430 mOsm	
	25°	37°	25°	37°
Hb AA	82 ± 4.7 (6)*	70 ± 4.8 (5)	107 ± 11.8 (4)	89 ± 21.8 (5)
Hb SS	135 ± 17.6 (7)	105 ± 9.5 (5)	124 ± 21.3 (5)	100 ± 19.5 (4)
Hb SA	83 ± 5.3 (3)	69 ± 8.9 (3)	128 ± 25.3 (3)	105 ± 28.6 (3)

* Values in parentheses are the number of experiments.

Table 2. Effect of the presence of ISC on $t_{1/2}$ (msec)

Fraction	% ISC	25°	37°
Top	3–20	113 ± 28.3	107 ± 0.7
Bottom	52–60	124 ± 6.4	112 ± 14.1
Control (unfractionated)	2–19	135 ± 19.7	105 ± 9.5

msec). The oxygenation rates for these two fractions were quite similar to those obtained with unfractionated samples of SS red cell at 25° and 37°.

Additional evidence for the role of the deoxy Hb S polymers in the kinetic behavior of SS red cells is presented in Fig. 2, which illustrates the effect of temperature variation (5–37°) on the rate of oxygenation of normal and SS red cells. It has been previously observed that the gel of deoxy Hb S liquifies (22) and red cell sickling is sharply reduced at low temperatures, indicating disruption of polymerization. Under the conditions of the present kinetic experiments, deoxygenation of SS red cells at 25° followed by warming to 37° for 45 min resulted in sickling of 81–85% of the previously normal-appearing red cells. Lowering the temperature of the deoxygenated cell suspension to 25°, 15°, and 12° produced little or no change in sickling, whereas at 4° the proportion of reversibly sickled cells fell to 12–21%. Excluding these low temperatures at which the deoxygenated SS cells became unsickled, the apparent heat of activation (ΔH^*) for the oxygenation of normal and SS red cells was obtained from an Arrhenius plot of the kinetic data between 11° and 37° (Fig. 3). The apparent heat of activation, ΔH^*, directly related to the slope of each plot, was nearly identical for the normal cells [2.86 kcal (11.97 kJ)/mole] and for SS cells [2.83 kcal (11.94 kJ)/mole] over this range of temperature.

The effects of addition of 0.1 M *n*-butylurea on the rates of oxygenation of normal and SS red cells at 25° and 37° are shown in Table 3. This antisickling agent (23) lowers the oxygenation $t_{1/2}$ of SS red cells from 133 ± 4.2 to 103 ± 2.8 msec at 25° and from 107 ± 11.3 to 78 ± 12.7 msec at 37°.

In contrast to the observed differences in the rates of oxygenation between normal and SS red cells, the kinetic constants for the *de*oxygenation reaction induced by dithionite (off constants) were indistinguishable (Table 4).

DISCUSSION

The present data show that the oxygenation time of fully deoxygenated SS red cells is distinctly prolonged as compared with that of normal red cells. The question arises—what are the

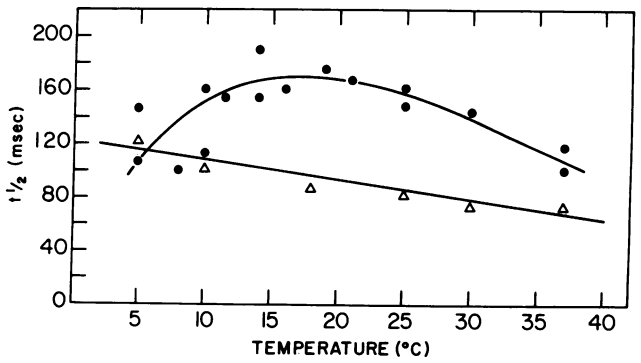

FIG. 2. The effect of temperature on the rate of oxygenation ($t_{1/2}$ msec) of normal (△) and sickle (●) red cells suspended in 0.15 M potassium phosphate, pH 7.35. Oxygen concentration (before mixing), 0.21 mM.

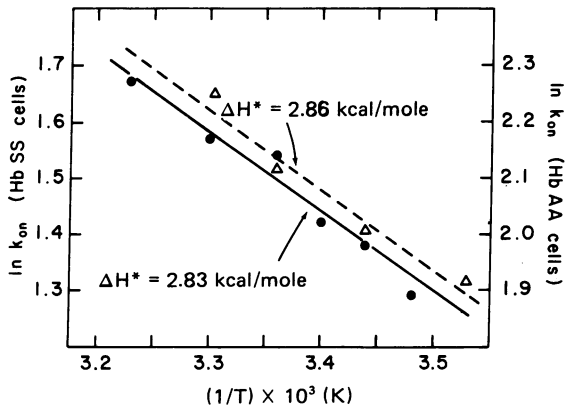

FIG. 3. Arrhenius plots for the apparent heat of activation (ΔH^*) for the oxygenation of normal (△) and sickle (●) red cells. Slope = $-\Delta H^*/R$, when R is the gas constant. Red cells were suspended in 0.15 M potassium phosphate, pH 7.35. Experimental data points in the range where no change in the extent of sickling and deformity of red cells were used to determine ΔH^*.

mechanisms of the observed differences? The two possibilities that seemed to be the most likely were (*i*) restraints to oxygenation resulting from the polymerization of deoxy Hb S and (*ii*) interference with passage of oxygen through the red cell membrane due to membrane abnormalities known to occur with SS red cells (and presumably most severe among ISC). Many of the experiments presented here were designed to help distinguish between these two possible mechanisms.

The evidence can be summarized as follows: (*a*) The oxygenation rate for sickle trait (SA) red cells suspended in isotonic buffer at pH 7.35 was equal to that of normal cells; under these conditions, the cells have been observed to undergo very little sickling, which occurred only when deoxygenation was nearly complete (21, 24). Raising the mean intracellular Hb concentration of SA red cells by osmotic shrinkage, however, was shown by Bookchin *et al.* to both increase their sickling propensity and lower their oxygen affinity to an extent equivalent to those of SS red cells (21). Correspondingly, suspension of SA red cells in such a hypertonic medium (430 mOsm/kg of H_2O) was accompanied by a slowing of their rate of oxygenation to that shown by SS red cells (Table 1). It should be noted that irreversibly sickled forms, with associated membrane abnormalities, are not found among SA red cells. Our finding that suspension of SS red cells in hypertonic media produced no further decrease in oxygenation rates suggests that in these cells, the effects of polymerization of the deoxy Hb on oxygenation kinetics are maximal at normal mean intracellular Hb concentrations. This is not surprising, for both in isotonic and hypertonic solution, SS cells exhibit maximal morphological sickling at oxygen saturations up to about 50%. In addition, the absence of difference in oxygenation rates between the light and dense (ISC-rich) fractions of SS cells is consistent with this interpretation. (*b*) If the polymerization of Hb within SS red cells were related to their slow rates of oxygenation, then we

Table 3. Effect of 0.1 M butylurea on the rate of oxygenation ($t_{1/2}$ msec) of Hb AA and Hb SS red cells

	AA cells		SS cells	
	25°	37°	25°	37°
Control	84 ± 5.2	72 ± 7.8	133 ± 4.2	107 ± 11.3
0.1 M Butylurea	92 ± 10.6	86 ± 12.7	103 ± 2.8	78 ± 12.7

Table 4. Rates of deoxygenation of Hb AA and Hb SS red cells by dithionite

Red cells	25°		37°	
	$t_{½}$ (msec)	k_{off} (sec^{-1})	$t_{½}$ (msec)	k_{off} (sec^{-1})
Hb AA	127.5 ± 17.7	5.49 ± 0.75	52.5 ± 0.7	13.2 ± 0.14
Hb SS	119 ± 12.7	5.85 ± 0.63	51.5 ± 7.7	13.7 ± 2.05

could expect the oxygenation rates of SS red cells to approach those of normal red cells at low temperatures. This effect should be expected because at temperatures less than 10° the polymerization of deoxy Hb S is inhibited. In fact, when the temperature was reduced to 5° the oxygenation $t_{1/2}$ for SS red cells became identical with that of normal cells (Fig. 2). The apparent larger difference in the $t_{1/2}$ between normal and sickled cells at 15° than at 37° is not reliable if the error of the determinations is taken into account. Future experiments will have to decide if this difference is real. (*c*) The absence of a difference in the heat of activation, in spite of difference in oxygenation rates, suggests that an entropy factor could be invoked to explain the differences between SS and normal cells. (*d*) Another line of evidence can be derived from the effect of *n*-butylurea, an agent that inhibits sickling (at 0.1 M concentration) without modifying the oxygen equilibrium curve of normal red cells (23). As can be seen in Table 3, *n*-butylurea did not affect the rate of oxygenation of normal cells, but in the case of SS cells, oxygenation rate was increased ($t_{1/2}$ lowered) to the range of normal cells. (*e*) Furthermore, the fact that the differences found between SS and normal cells were restricted to the oxygenation rates while their deoxygenation constants (with dithionite) were identical also supports the notion that polymerization of deoxy Hb S is the main factor involved in the changes of oxygenation kinetics. Moreover, these findings established that the low oxygen affinity exhibited by Hb S-containing red cells under these conditions results mostly from the decreased rate of *uptake* of oxygen by sickled cells and is largely independent of the rate of oxygen egress.

We can conclude that the data as a whole strongly suggest that the slowing of the oxygenation reaction exhibited by SS red cells results largely, if not exclusively, from the polymerization of deoxy Hb S. It is possible that the restraints introduced by the polymer stabilize the quaternary deoxy conformation of the tetramers (the "T state") or generate an altered quaternary form (T* state) with decreased affinity for oxygen. Finally, the decreased fluidity of the cell contents may impede convective or Hb-facilitated movement of oxygen through the interior of the cell.

Note Added in Proof. After the submission of this paper, the work of Rotman *et al.* [*Respir. Physiol.* (1974) **21**, 9–17] has come to our attention. These authors find, as we do, a difference in the rate of oxygenation between SS and normal red cells. They also observed, in contrast to our findings, a difference in the rate of deoxygenation, concluding that these effects did not result from the intrinsic properties of the two hemoglobins but from intracellular environmental factors. Our data appear to contradict these conclusions.

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