Oxygen binding by single red blood cells from the red-eared turtle *Trachemys scripta*

SEBASTIAN FRISCHE,1 STEFANO BRUNO,2 ANGELA FAGO,1 ROY E. WEBER,1 AND ANDREA MOZZARELLI2

1Danish Centre for Respiratory Adaptation, Department of Zoophysiology, University of Aarhus, 8000 Aarhus C, Denmark; and 2Institute of Biochemical Sciences, University of Parma, Area Parco delle Scienze, 43100 Parma, Italy

Received 8 August 2000; accepted in final form 2 December 2000

Frische, Sebastian, Stefano Bruno, Angela Fago, Roy E. Weber, and Andrea Mozzarelli. Oxygen binding by single red blood cells from the red-eared turtle *Trachemys scripta*. J Appl Physiol 90: 1679–1684, 2001.—Oxygen-binding properties of single red blood cells from the red-eared turtle *Trachemys scripta* were measured by microspectrophotometry to describe the variation in oxygen affinity of red blood cells and to gain insight into the distribution of functionally different hemoglobins among red blood cells. Methodologically, this study represents the first report on the cell-to-cell variation in oxygen-binding properties based on oxygen-binding curves of single vertebrate red blood cells. The cells differed significantly with respect to oxygen affinity. Mean oxygen pressure at half saturation of the cells in a blood sample was found to be 20.1 ± 3.3 (SD) Torr. The distribution of oxygen affinities among red blood cells is unimodal, indicating that the two hemoglobins found in turtle blood are not segregated in distinct cells. Therefore, the functional interaction shown by these hemoglobins in vitro is likely to take place in vivo. The considerable variation in oxygen affinity between individual red blood cells calls for its incorporation in models of tissue oxygenation.

Although the description of vertebrate red blood cells as “bags of hemoglobin” is often a useful generalization, these very specialized and simplified cells may show significant variability in their biological function as oxygen carriers. This variability allows red blood cells to continuously adjust their properties in response to changes in factors like oxygen tension (PO2) (27), carbon dioxide tension (PCO2), osmolality, temperature, season (21), and concentration of hormones (14, 31).

In contrast to that of humans, the blood of most vertebrate species contains several types of hemoglobin even in the adult state, thereby adding the distribution of hemoglobins among red blood cells to the list of factors that contribute to red blood cell variability. Moreover, the majority of vertebrates (e.g., fish, amphibians, reptiles, and birds) have nucleated red blood cells, which show high aerobic metabolic rates (22) and are capable of protein synthesis during circulation (30), making these cells even more complex than mammalian red blood cells.

Two functionally different hemoglobins can be separated from the blood of the adult red-eared freshwater turtle *Trachemys scripta* (11). The hemoglobins differ in oxygen affinity, expressed as oxygen pressure at half saturation (P50): 5.6 and 4.0 Torr (25°C, 100 mM HEPES, pH 7.3, and 100 mM KCl); moreover, there is a marked functional interaction in vitro. A 1:1 mixture of the two hemoglobins at similar conditions has a P50 value of 10.0 Torr. Cooperativity [measured by the slope of the Hill plot (n)] changes were from 2.2 and 2.4 in the isolated hemoglobins to 2.0 in the 1:1 mixture (Frische, unpublished observations). For algebraic reasons, a mixture of noninteracting hemoglobins with different P50 values will result in a reduced n (12). However, an increase in P50 values is unexpected and indicates functional interaction between the hemoglobins. A prerequisite for this functional interaction to take place in vivo is the presence of both hemoglobins in the same cells. Because the hemoglobins show different affinities for oxygen, the distribution of hemoglobins among red blood cells will be reflected in the corresponding distribution of oxygen affinities.

Apart from hemoglobin composition, the age profile of the circulating red blood cells affects cell-to-cell differences in oxygen affinity. Red blood cells are continuously formed and removed from the blood; the mean red blood cell life span in mammals is generally dependent on body weight, with the larger species having cells with longer life spans (33). Therefore, the cell-to-cell differences within a red blood cell population are often related to the activity of the different erythropoietic tissues, which may modify both the age profile of the circulating cells and their biochemical characteristics, e.g., hemoglobin composition (20). The red blood cells of turtles live for up to 11 mo (15) and may exhibit large age-related diversity. Moreover, turtle red blood cells have been proposed as a model for the evolutionary transition state between red blood
cells relying on aerobic metabolism and the anaerobi-
cally metabolizing mammalian red blood cells (18), a
transition that is homologous to that occurring in ma-
turing mammalian red blood cells.

To map the distribution of hemoglobins and to de-
scribe the variation of oxygen affinity in these long-
living red blood cells, we measured oxygen-binding
properties by microspectrophotometry of individual
turtle red blood cells. All available methods for mea-
suring blood oxygen dissociation curves measure mean
values for a large number of cells (11). The possibility
to obtain oxygen-binding curves on single cells by mi-
crospectrophotometry has been acknowledged for more
than a decade (4, 17), but, to our knowledge, the tech-
nique has not been applied to vertebrate red blood
cells. By determining oxygen affinity and cooperativity
for individual cells, we can provide descriptions of the
variation of these main functional properties in a pop-
ulation of nucleate red blood cells.

MATERIALS AND METHODS

Blood samples were taken with heparinized syringes from
the coccygeal vein (26) of adult *Trachemys scripta* kept at
25°C. The blood was directly transferred to two gas-tight
vials preequilibrated with 95% air and 5% CO₂. One vial was
centrifuged for 5 min at 1,500 rpm to separate blood cells
from plasma. The plasma was removed and centrifuged at
10,000 rpm for 5 min to remove all other cellular and partic-
ulate matter. Less than 1 µl of blood from the second vial was
diluted with ~200 µl of plasma. A drop of this dilute cell
suspension was loaded in a Dvorak-Stotler flow cell (9),
which was covered by a gas-permeable silicon copolymer
membrane. The red blood cells settled one by one on the glass
bottom and remained stationary for hours. The flow cell was
mounted on the thermostated stage of a Zeiss MPM03 mi-
crospectrophotometer (24). The light transmitted through the
red blood cell, and the surrounding solution was recorded
using a circular measuring spot of ~8 µm in diameter. The
spot was smaller than the average size of a single ellipsoid
turtle red cell (19 × 11 µm) (25). Spectra were recorded in the
wavelength range 380–450 nm at 1-nm intervals.

Gas mixtures consisting of 5% CO₂, 2.1–4.8% O₂, and
92.9–90.2% He were prepared as follows. O₂ and He were
mixed in discrete steps with a gas-mixture generator (Envi-
ronics, series 200). The output gas was then mixed with He
and CO₂ by a gas mixing pump (Digamix M301/a) to obtain
5% CO₂ in the final gas mixture. The final mixture was
humidified by bubbling in a 0.9% NaCl solution and passed
through the flow cell. The equilibration of the system to a
new PO₂ required ~20 min. All the experiments were carried
out at 25°C and within 6.5 h after blood sampling.

Absorbance spectra of fully oxygenated and deoxygenated
red blood cells were recorded after equilibration with 5%
CO₂-95% O₂ and 5% CO₂-95% He, respectively. The frac-
tional oxygen saturation of hemoglobin was estimated by
fitting a linear combination of these reference spectra and a
baseline correction consisting of a horizontal offset and a
variable slope to the observed absorbance spectra at inter-
mediate PO₂ levels (23). The standard deviation of the fitted
fractional saturation was estimated from the variances cal-
culated in the fitting procedure and subsequent error propa-
gation calculations (29). Data are given as means ± SD
unless otherwise stated.

RESULTS

Oxygen saturation of a single cell. Four spectra of a
single red blood cell at different oxygen pressures are
presented in Fig. 1A. Two spectra were obtained at the
same oxygen pressure (35.1 Torr), at the beginning and
the end of the experiment, respectively. The two spectra
gave nearly identical fractional saturation values of
0.641 ± 0.011 and 0.645 ± 0.007, illustrating the reversibility
of the oxygenation and the stability of the
cell preparation during the oxygen-binding experi-
ment, which typically lasted 5 h. The absorbance spec-

![Fig. 1. A: absorbance spectra of a single red blood cell exposed to PO2 values of 35.1, 25.2, 15.4, and back to 35.1 Torr. B: comparison between the observed absorbance spectrum at 19.7 Torr O2 and the fitted spectrum obtained from a linear combination of the oxy- and deoxy-reference spectra and baseline correction. Fractional oxygen saturation is estimated to be 0.472 ± 0.007 and root mean square 0.000017. The difference in absorbance (Δ) between observed and calculated spectrum (dashed line) refers to the right y-axis.](http://jap.physiology.org/Downloadedfrom/10.1152/jappl.00230.2000)
tra are not identical because the position of the measuring spot is not the same, which reflects the variable thickness of the red blood cells.

Figure 1B shows the observed spectrum at 19.7 Torr O₂ with the spectrum calculated by linear least squares fitting, giving a mean fractional saturation of 0.472. The SD of the mean fractional saturation was small (0.0096 ± 0.0039 (mean ± SD of 110 saturation measurements)), and the predicted 95% confidence interval of fractional saturation was ±0.02. To verify the accuracy of these intervals, the fractional saturation of two cells were measured repeatedly (9 and 11 times) at constant PO₂ (see Fig. 2). These cells had fractional saturations of 0.68 ± 0.016 and 0.64 ± 0.012, resulting in 95% confidence intervals of ±0.038 and ±0.027, respectively. These intervals are narrower than those reported from another microspectrophotometric system (10). This difference can probably be ascribed to our use of 11 absorbance values from the entire absorbance spectrum from 380 to 450 nm instead of the difference in optical densities at only two wavelengths. This fitting procedure significantly increases the overall precision of the measurement.

Oxygen saturation in a red blood cell population. The variability of red blood cell oxygen affinity is evident from the distribution of fractional saturations of 38 cells at a fixed oxygen pressure of 25.2 Torr (Fig. 2). The mean fractional saturation was 0.595 ± 0.055, and the corresponding 95% confidence limits were 0.482 and 0.707. The SD of the population data was about four times that of the repeated measurements of individual cells (see above). Thus it cannot be ascribed to measuring error but must be considered a reliable estimate of the intercellular variation. On the basis of an n value of 1.71 (6), these data indicate that the mean P₅₀ value for this cell population was 20.1 ± 3.3 Torr and the values of the two cells subjected to repeated measurements were 17.8 ± 0.6 and 16.1 ± 0.7 Torr. The distribution of saturations of the 38 cells is qualitatively unimodal. Assuming a normal distribution, we obtained a χ² value of 3.316, which is far from rejecting the null hypothesis of normality (0.7 < P < 0.95) (34).

Oxygen binding of single cells. Hill plots of seven individual cells from two different turtles are presented in Fig. 3, and the corresponding seven sets of P₅₀ and n values are shown in Table 1. Table 2 presents the details of an analysis of covariance (ANCOVA) of the data from each sample. The ANCOVAs show the elevations of the regression lines within each sample to be significantly different (P < 0.0001 in both samples), whereas the slopes of the plots (n) are not (P > 0.75 in both samples) (35). This implies that the oxygen affinity differs significantly between the red blood cells of a given blood sample. Mean P₉₀ values of the two samples of cells are 20.4 ± 2.3 and 20.9 ± 1.3 Torr, and the mean n values of the two samples are 1.52 ± 0.14 and 2.13 ± 0.09.

DISCUSSION

This study represents the first report on cell-to-cell variation in oxygen affinity and binding cooperativity of vertebrate red blood cells. Literature values for P₅₀ and Hill’s n are averages of many cells and may only be compared with the mean values of our single cell measurements. The average P₅₀ values found in this study, 20.4 ± 2.3 and 20.9 ± 1.3 Torr, are close to the value of 24.5 Torr reported for whole blood studies of Trachemys scripta at similar temperature and PCO₂ values (6). Similarly, the mean n values, 1.52 ± 0.14 and 2.13 ± 0.09.
0.09, are within the range of earlier reported values for *Trachemys scripta* and other cheloniens (6). No directly comparable data exist for the variance of P50 between cells or the distribution of fractional saturations at fixed PO2 reported in this study. From a technical point of view, the only directly comparable study concerns the blood cells of the annelid worm *Glycera dibranchiata* (17), in which case the P50 values at pH 7.4 and 20°C were 11.5 ± 1.08 and 5.4 ± 0.70 in the absence and presence of 20 mM KCN, respectively.

Microspectrophotometry has previously been used to monitor fractional oxygen saturation of single red blood cells from trout to describe the distribution of hemoglobin with a root effect among red blood cells (5). The span of fractional saturations of individual cells at fixed pH and PO2 was shown to be −0.12 in trout (from Fig. 1 in Ref. 5), but no statistics were given. In comparison, the range of saturations in Fig. 2 is 0.26. Microspectrophotometric techniques have also been applied to monitor the kinetics of reversible binding of CO and O2 to hemoglobin in single red blood cells (1, 2) and in a study of the diffusion of gases into red blood cells (8). In contrast to all prior microspectrophotometric studies, in which washed cells were resuspended in buffer to control pH, the cells used in this study were kept in natural plasma buffered with CO2. The resultant data are thus directly comparable to traditional whole blood measurements.

Table 1. P50 and Hill’s n calculated from the Hill plots in Fig. 3

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<th>Turtle a</th>
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<tbody>
<tr>
<td>P50</td>
<td>20.6</td>
<td>18.6</td>
<td>23.6</td>
<td>18.9</td>
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<tr>
<td>n</td>
<td>1.38</td>
<td>1.56</td>
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Symbols are from Fig. 3. P50, O2 pressure at half saturation.

Although a bimodal distribution cannot be completely excluded, the distribution of saturations (Fig. 2) appears unimodal and normal (0.7 < P < 0.95), indicating that the two functionally different hemoglobins (11) are not separated in distinct cells. Minor differences in the proportion of the two hemoglobins may influence the variance of the distribution of P50 values among cells. In this respect, the results confirm the expression pattern of globin genes described for other adult vertebrates (5). Because differences in hemoglobin constitution of the red blood cells are unlikely to be involved, we must look for other factors to explain the significant differences in oxygen-binding properties of red blood cells found in this study.

Cell age is the major generator of variance in red blood cell population parameters and is therefore a prime candidate for causing the observed cell-to-cell variance in oxygen affinity. Human red blood cells undergo gradual biochemical changes during their ~120-day life span (7), such as loss of K1, decreased enzyme activity, decreased 2,3-diphosphoglycerate (DPG) concentration, and increased hemoglobin concentration. These changes are accompanied by reduced cell deformability and increased density. The latter feature can be exploited in the traditional density-gradient centrifugation for separating red blood cells in age fractions (7). Similar age-dependent changes are shown in red blood cells from other mammals (33) and...
in lower vertebrates, despite differences due to the presence of aerobic metabolism and hemoglobin synthesis during circulation (22, 30).

In humans, the oxygen-binding properties of old red blood cells separated by centrifugation are characterized by a higher affinity for oxygen compared with young cells. The difference is mainly associated with decreasing erythrocytic DPG concentration with cell age (28). Other factors like glycosylation of hemoglobin and formation of methemoglobin in the cells may also contribute to the increased oxygen affinity of old red blood cells (28). We suggest that the differences in oxygen-binding properties between red blood cells documented in this study are paralleled by differences in the concentration of ATP, which is the dominant organic phosphate compound in Trachemys red blood cells (3) and which modifies the oxygen affinity of both hemoglobins (11). Turtle red blood cells have a much longer life span than their human counterparts; therefore, the magnitude of the age-dependent intercell variation in oxygen affinity is probably larger than in humans, although no conclusions can be drawn on the basis of the available data.

Blood generally behaves as a homogeneous liquid with respect to oxygen transport, but, in the capillaries, oxygen diffuses to the surrounding tissue from cells that move in single file. Variation in oxygen affinity affects the amount of oxygen released from each red blood cell for a given decrease in PO2. The cell-to-cell variation in oxygen-binding properties may therefore play a physiological role by influencing tissue PO2 gradients and, at least locally, tissue oxygenation levels. Modeling has been an important constituent in the study of tissue oxygenation since the beginning of the last century. An extensive literature has grown from the original Krogh model (16), generally expanding the Krogh cylinder to deal with the effects of the particulate nature of blood, the shape of erythrocytes, variations in hematocrit, uneven temporal distribution of red blood cells, and anisotropic distribution of capillaries (13, 32, 34). In all models, P50 is assumed to be constant for all red blood cells; however, from our results, it is clear that considerable variation exists between individual red blood cells with respect to oxygen affinity. This study therefore calls for modeling studies that include the consequences of variations in red blood cell oxygen affinity with respect to tissue oxygenation.

Oxygen equilibria of single red blood cells may also prove useful in other areas of research. In diseases in which the erythrocytes are differentially affected, e.g., malaria, single cell measurements would be able to quantify the effects of oxygen binding on the cellular level. In the case of malaria, the oxygen binding of blood cells containing different stages of the parasite could be quantified. Because only a few microliters of blood is necessary, single cell oxygen binding could also be used to study variations in blood oxygen binding within natural populations of small animals.

Another field in which the determination of oxygenation curves of single red blood cells may be useful is the testing of drugs affecting oxygen affinity. Agents that increase the oxygen affinity are of therapeutic benefit in sickle cell anemia, which is caused by the polymerization of deoxygenated sickle hemoglobin. The polymerization induces changes in the shape of the red blood cells and shortens their circulatory survival, resulting in severe anemia. The increase of oxygen affinity reduces the amount of the insoluble deoxygenated sickle hemoglobin in venous capillaries, thus preventing red blood cells from sickling. Previous microspectrophotometric studies monitored the kinetics of sickle hemoglobin polymerization within single red blood cells and determined the distribution of sickling cells as a function of oxygen saturation (19). Microspectrophotometry could be used to monitor both affinity changes and morphological alteration of single cells in response to drugs.

We thank the Danish Natural Science Research Council and the Italian National Research Council for financial support and Academia di Danimarca, Roma, for providing housing for turtles.

REFERENCES


