Small Angle X-ray Scattering (SAXS) Study of the Extracellular Hemoglobin of Glossoscolex paulistus

EFFECT OF pH, IRON OXIDATION STATE, AND INTERACTION WITH ANIONIC SDS SURFACTANT*

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Annelid extracellular hemoglobins are giant molecules that have a characteristic hexagonal bilayer appearance in electron micrographs, high cooperativity of oxygen binding, and low iron and heme contents (1–4). The hemoglobin of the earthworm Lumbricus terrestris (HbLt)3 is the most extensively studied annelid hemoglobin. It consists of about 180 polypeptide chains in an arrangement of two heme-containing subunits (monomer M and disulfide-bonded trimer T) and non-globin linker subunits with molecular masses in the range 24–32 kDa. A model of quaternary structure (bracelet model) explains the properties and subunit structure of HbLt: three copies of monomer subunit and three copies of the trimer subunit form a docosamer subunit, (abcd)3, of about 200 kDa. Twelve such complexes of heme-containing chains are linked together by 36 heme-deficient linker chains to provide the total mass of about 3500 kDa. The 1/12 subunit of the whole protein is associated then to (abcd)3L12, where L stands for the linker chains. Since 1996 a significant effort has been devoted by several laboratories to elucidate in more detail the arrangements between the subunits from the structural point of view to better understand the oxygenation of this giant hemoglobin. The three most important contributions in this research are associated with the mass spectrometry determination of the molecular masses of all subunits (3), to the three-dimensional reconstructions at a low resolution of 35 Å obtained by cryoelectron microscopy (1) and which were able to give results on the shape and dimensions of the whole protein, and, finally, to the recently reported crystal structure of Hb of L. terrestris obtained at a resolution of 5.5 Å, which allowed us to establish a complicated hierarchy of overall D6 symmetry, in agreement with previous findings, and local D3 symmetry for smaller subunits as well as the location of the linker chains in the whole oligomeric structure (5).

The extracellular hemoglobin of Glossoscolex paulistus (HbGp) is a giant hemoglobin, similar to other annelid hemoglobins, especially to L. terrestris Hb, and is also constituted by a large number of subunits containing heme groups with molecular masses in the range of 12–16 kDa forming a monomer of 16 kDa and a trimer and non-heme structures, namely linkers, with molecular masses in the range 24–32 kDa, conferring to the whole protein a molecular mass near 3100 kDa and a double layered hexagonal oligomeric structure (6, 7). In Fig. 1 a schematic model is shown for the hexagonal bilayer structure of HbGp and HbLt based on the available experimental data, mostly for HbLt, as mentioned above. For HbGp the full amino acid sequence is known only for the monomer chain d (8), although evidence exists for a great similarity of its oligomeric structure with that for HbLt.

Fluorescence studies of HbGp have shown that the dissociation of the oxy-form of Hb of G. paulistus at alkaline pH 9 is not complete, resulting in the (abcd) fraction as the main product. Changes in the fluorescence for the intact oligomer as well as for the monomer, trimer, and the (abcd)3 fractions can be compared, showing quantum yields consistent with data from other hemoproteins, where a significant quenching due to energy transfer from tryptophans to hemes occurs (9, 10). The oxidation of the iron with the production of the met-form of hemoglobin leads to a complete dissociation of the oligomer at pH 9 into its constituent small molecular mass subunits (linkers,
The SDS technique has been used as a powerful tool in the study of proteins, since important parameters such as size and shape can be obtained for the macromolecule (29–32). Small angle scattering data treatments have been developed for the purpose of extracting the maximum information about the systems, especially in the case of proteins (33), where conformation details can be retrieved from the SAXS data by means of different models of densely packed beads. Those are obtained from the correspondent pair distance distribution functions (p(r)). Previous SAXS studies of HbLt have reported the dimensions of the whole molecule as well as the dodecameric subunit (abcd)4, and one of the relevant results was the fact that the oxygenation did not induce shape or dimension alterations (34).

MATERIALS AND METHODS

The hemoglobin of G. paulistus was prepared by a centrifugation at 4 °C (2500 rpm for 15 min) followed by an ultrafiltration (molecular mass cut-off 30 kDa) and ultraconcentration at 250,000 × g at 4 °C during 3 h. The Hb is obtained as a pellet and then resuspended in a minimum amount of 0.1 M Tris-HCl buffer, pH 7.0, and stored in the oxy-form at 4 °C. Chromatography at pH 7.0 in a Sephadex G-200 column furnished the samples used in the experiments. The met-form of the hemoglobin was obtained adding an aliquot of ferricyanide stock (0.1 M) to oxyhemoglobin solution followed by filtration in a Sephadex G-25 column to remove the excess of ferricyanide. All concentrations were determined spectrophotometrically in a Hitachi U2000 spectrophotometer using the appropriate molar extinction coefficients (9, 10) to obtain a final protein concentration of 3 mg/ml in 20 mM Tris-HCl buffer. The samples used for SAXS experiments were prepared, in general, 24–48 h prior to the measurements. For samples with surfactant, addition of aliquots of concentrated SDS solutions to the 3 mg/ml protein solution was made to give the desired final SDS concentration. Experiments with samples prepared immediately before the measurements resulted in similar scattering curves suggesting that the equilibrium of different species in solution was reached in a relatively short time.

SAXS experiments were performed at the National Synchrotron Light Laboratory, Campinas, Brazil, using a detector-sample distance of 731 mm. The scattering vector amplitude q is defined as 
\[ q = \frac{4\pi}{\lambda} \sin(\alpha/2) \]

where \( \alpha \) is the scattering angle and \( \lambda \) the x-ray wavelength of 1.60 Å. The experimental intensities were corrected for sample attenuation, detector homogeneity, and blank scattering. For protein samples containing up to 2 mM SDS, the blank contribution consisted of the surfactant-free buffer solution scattering; once up to such surfactant concentration the scattering curves are identical to that ofbuffer solution. For the 20 mM SDS-containing HbGp sample, the buffer solution scattering curve was also considered as a blank contribution, because the scattering curve for the protein-surfactant system presented the characteristic scattering of SDS micelles with a broad peak positioned at 0.19 Å⁻¹, while such peak appears at 0.15 Å⁻¹ for protein-free SDS micelles solution (35). For this reason no attempt was made to subtract the scattering curve of 20 mM surfactant, since the difference in peak positions in these two systems, pure micelles and protein micelles, would lead to artifacts in the corrected protein/surfactant scattering.

It is well known that the scattering intensity from a set of monodisperse particles randomly distributed, in the absence of interparticle interference function, is given as,

\[ I(q) = kP(q) \]  

(Eq. 1)

where \( k = \gamma / (\Delta \rho) \), \( \Delta \) is the density contrast between the scattering particle and the medium, and \( V \) is the scattering particle volume. \( P(q) \) in Equation 1 is the normalized particle form factor \( P(0) = 1 \).

A Fourier transform connects \( P(q) \), and hence \( I(q) \), to the p(r); the probability of finding a pair of small elements at a distance r within the entire volume of the scattering particle is given as follows (36, 37).
This function provides information about the shape of the scattering particle as well as its maximum dimension, \( D_{\text{max}} \), accounted for at a certain \( r \) value where \( p(r) \) reaches zero. Moreover, the particle radius of gyration \( R_g \) value is defined as follows (37).

\[
p(r) = \frac{1}{2\pi^2} \int_0^\infty I(q)q \sin(qr) dq
\]  
(Eq. 2)

\[
R_g^2 = \frac{\int_0^{R_{\text{max}}} p(r)r^2 dr}{2 \int_0^{R_{\text{max}}} p(r) dr}
\]  
(Eq. 3)

In this work, we make use of the GNOM program (38) to calculate \( p(r) \) from the SAXS curves.

The \( R_g \) value can also be evaluated from the scattering curve at low \( q \) range where the intensity obeys Guinier’s law (36),

\[
I(q) = k \exp(-R_g q^2/3) (q \rightarrow 0)
\]  
(Eq. 4)

so that \( R_g \) can be directly calculated from the slope of the linear part of \( \ln I(q) \) versus \( q^2 \) plot. This approximation is valid for values of \( R_g \) such that \( qR_g < 1 \).

It should be remarked that it is possible to measure the intensity in absolute scale in a SAXS experiment (39, 40; \( \gamma \) is determined) and, hence, to obtain information about the prefactor \( n_o \langle \Delta \sigma \rangle V^2 \) that is directly related to the scattering particle molecular mass at the initial scattering intensity \( I(0) \) (35, 37). In this work, we were just interested in investigating the differences in the macromolecule scattering functions as a function of added surfactant in relation to the system in the absence of SDS, at different pH values. Therefore, no absolute scale measurement was done at this stage, in such a way that \( k \) was used as a normalization factor. Nevertheless, a qualitative comparison and interpretation of the extrapolated \( I(0) \) values will be offered into the discussion of the results.

An interesting procedure was used to determine the protein shape through a model described by densely packed beads (33). Such a method restores the low resolution of biological macromolecules in solution by using simulated annealing. This program uses as an input file the \( p(r) \) function given by the GNOM program. For details see Ref. 33. An initial symmetry for the model is generally required, because according to theoretical analysis of SAXS curves, the information content is limited by the number of degrees of freedom (Shannon channels) contained in the experimental data (41). The introduction of symmetry constraints increases the number of degrees of freedom and, consequently, leads to the restoration of a better three-dimensional model (42).

RESULTS

Fig. 2 shows the scattering data of the oxy-HbGp at pH 7.0 in the absence and presence of different SDS concentrations. For detergent-free and 0.2 mM SDS-containing (curve not shown) protein solutions at pH 7.0, the SAXS curves are characterized by two well defined shoulders in the \( q \) region of 0.04 Å\(^{-1}\) and 0.07 Å\(^{-1}\) and a third one, not so pronounced, in the \( q \) region of 0.12 Å\(^{-1}\). These shoulders are characteristic of extracellular giant hemoglobins (4). Variation of protein concentration in the absence of SDS the observed 6-fold symmetry (Fig. 1A), a radius of gyration \( R_g \) value obtained from the beads model. The SAXS curve of oxy-HbGp at pH 7.0 under the influence of 2 mM detergent (Fig. 2A) does not present the characteristic shoulders (they are not so clear), and the extrapolated initial scattering intensity decreases 2.5-fold as compared with the curve of the protein in the absence of SDS (Fig. 2A). These data are compatible with the fact that the whole protein could dissociate separating initially into two hexagonal layers. Fig. 3, A and B, show the scattering data (open symbols) of oxy-HbGp solution at pH 7.0 in the absence and presence of 2 mM SDS, respectively, along with theoretical curves (solid lines) associated with the corresponding \( p(r) \) functions (normalized) shown in Fig. 3C. The \( R_g \) and the \( I(0) \) obtained from \( p(r) \) are included in

Table I. The values of \( R_g \) ~ 110 Å and \( D_{\text{max}} \) ~ 300 Å (Fig. 3C) obtained for oxy-HbGp in the absence and presence of 0.2 mM SDS are similar to those obtained through the SAXS technique for the extracellular hemoglobin of \( L. \) terrestris, where \( R_g \) = 107–114 Å and \( D_{\text{max}} \) = 294 Å (2, 34). It is worthy to emphasize that the normalized \( p(r) \) curves present the same value of scattering particle maximum dimension \( D_{\text{max}} \) (Fig. 3C), although \( I(0) \) decreases almost a factor of 2.5 when 2 mM surfactant is added to the protein solution. The densely packed bead models are presented in Fig. 3D. It should be remarked that these models were obtained following two steps; in the first one, no symmetry was imposed, and the result showed a figure with 6-fold symmetry. Then, such symmetry was imposed, and the final model converged to the same structure. In the case of the protein solution in the absence of SDS the observed 6-fold symmetry (top view in Fig. 3D) may correspond to an array of protein subunits forming two hexagonal layers (Fig. 1A), which is in agreement with the symmetry, extensively revealed in electron microscopy studies (1, 2, 43, 44). From the top and lateral views (Fig. 3D), the estimated dimensions are 272 and 271 Å, respectively. In fact, assuming that the whole protein molecule can be considered as a cylinder-like (height \( L \) and radius \( R \)) model (Fig. 1A), a radius of gyration \( R_g^2 = R^2/2 + L^2/12 \) (36, 37) approximately 124 Å is obtained, taking into account the dimensions estimated from the beads model. The experimental \( R_g \) value obtained from \( p(r) \) analysis is approximately 12% smaller, probably due to the considered theoretical cylinder model that is just an approximation of the protein structure. The latter (Fig. 1A) contains some cavities that must contribute to the reduction of the \( R_g \) value. It is worth mentioning that these estimates are quite similar to the dimensions for HbLt obtained from three-dimensional reconstruction of cryo-microscopic images where values of 308 and 288 Å were obtained for the top and lateral views, respectively (44). In the presence of 2 mM SDS the top view dimension is maintained,
in buffer solution \((/H18554)\) to 96 Å deform the 6-fold symmetry. The lateral dimension is reduced to a 2-fold one. The presence of bound SDS could also smear or dissociation process, in agreement with a 2.5-fold decrease instead of due to a substantial loss of mass related to the protein dissociation into smaller subunits takes place. The \(p(r)\) analysis was not done for this particular case, because the scattering curve also presents a broad intramicellar peak that can lead to some misleading in the protein \(R_g\) determination by \(p(r)\) function.

A control experiment was performed monitoring the fluorescence of oxy-HbGp at pH 7.0 (data not shown) under the addition of increasing SDS concentration. Up to 1.5 mM surfactant the total fluorescence increased almost twice, whereas 8- and 11-fold fluorescence increases were detected in the range of 2–6 mM and 20 mM SDS, respectively. These results suggest that up to 2 mM SDS essentially surfactant binding occurs with very small HbGp dissociation, whereas above such surfactant concentration, protein/SDS binding, dissociation, and probably denaturation take place simultaneously. A direct comparison of fluorescence and SAXS data is not straightforward, since in fluorescence experiments protein concentration is kept at 0.12 mg/ml to avoid inner filter effect, while SAXS experiments were performed on 3 mg of protein/ml of solution. It means that the experiments probe different protein:surfactant molar ratios.

At pH 9.0, the SAXS curves obtained for oxy-HbGp in the presence of SDS up to 2 mM do not change as compared with the data of the detergent-free protein solution (Fig. 4A), revealing a \(R_g\) value around 33 ± 3 Å, from Guinier and \(p(r)\) analyses. Such value is similar to the value obtained when 20 mM SDS is added to the solution at pH 7.0 and 9.0 (Fig. 4B) (Table I). The scattering data do not present the characteristic shoulders of extracellular hemoglobins, and they do not differ from each other in the initial intensity and feature, suggesting that at pH 9.0, the dissociation of the protein takes place, independent of the surfactant concentration. The \(p(r)\) curve from the macro-molecule (similar to the curves of the protein interacting with

![Fig. 3. Experimental small angle x-ray scattering curves obtained at 25 °C in 20 mM Tris-HCl buffer at pH 7.0 for 3 mg/ml oxy-HbGp in: buffer solution (\(A\)) \((A)\), the presence of 2 mM SDS \((B)\) \((B)\), normalized \(p(r)\) \((C)\) \((C)\), and densely packed beads model obtained from \(p(r)\) curves with frontal \((left)\) and lateral \((right)\) view for oxy-HbGp buffer solution \((top)\) in and the presence of 2 mM SDS \((bottom)\) \((D)\). Continuous lines correspond to the best intensity fitting obtained from GNOM program to the experimental scattering curves.](http://www.jbc.org/)

**Table I**

| Buffer                  | Oxy-HbGp | Met-HbGp |
|-------------------------|----------|----------|
| pH 7.0                  | pH 7.0   | pH 9.0   |
| Buffer                  | 110 ± 10^5 | 35 ± 3^b | 29 ± 5^a |
| (0.533)                 | (0.013)  | (0.476)  |
| +0.2 mM SDS             | 110 ± 10^5 | 35 ± 3^b | 31 ± 3^b |
| (0.589)                 | (0.013)  | (0.546)  |
| +2 mM SDS               | 104 ± 10^6 | 33 ± 3^a | 65 ± 10^a |
| (0.222)                 | (0.012)  | (0.537)  |
| +20 mM SDS              | 24 ± 8^d | 36 ± 4^d | 27 ± 8^d |
|                         | 39 ± 5^a |          |

*Values were obtained directly from the Guinier plot (Equation 4) given in Å.

*Values were obtained from the distance distribution function \(p(r)\) through the GNOM program; values in parentheses correspond to the \(I(0)\) values obtained from the correspondent distance distribution function \(p(r)\).*

Although the 6-fold symmetry is less defined. This must occur due to a substantial loss of mass related to the protein dissociation process, in agreement with a 2.5-fold decrease instead of a 2-fold one. The presence of bound SDS could also smear or deform the 6-fold symmetry. The lateral dimension is reduced to 96 Å, supporting the hypothesis of the initial dissociation of the oligomer upon addition of 2 mM SDS at pH 7.0. Furthermore, \(R_g = 100\) Å is obtained by considering a cylinder-like model when \(L = 96\) Å is accounted for. Such a finding is consistent with the experimentally observed (Table I) \(R_g\) value.

At 20 mM SDS a drastic change is observed (Fig. 2B). The marked decrease in the initial intensity is accompanied by the disappearance of the characteristic shoulders of the experimental data. A broad peak in the region of 0.19 Å\(^{-1}\) is a fingerprint of the formation of SDS micelle-like aggregates in solution (35). The radius of gyration decreases to approximately 30 Å, obtained from Guinier analysis, suggesting that the whole protein dissociation into smaller subunits takes place.
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SDS up to 2 mM (Fig. 4A)) is presented in the Fig. 5B. Similarly, the beads model top view is shown in Fig. 5C, and the maximum dimension is estimated as 114 Å. Note that the small value of $R_g$ in comparison with that observed at pH 7.0 (33 ± 3 Å versus 107 ± 3 Å) is accompanied by a reduction of a protein maximum dimension $D_{max}$ from 300 Å to approximately 110–120 Å, indicating that at pH 9.0, the protein presents a low structural stability as compared with pH 7.0, dissociating into smaller subunits even in the absence of SDS. The dimensions and $p(r)$ function obtained for HbGp at pH 9.0 are very similar to those reported by Krebs et al. (45), which assume a dodecameric subunit for HbLt. However, in our case, from the I(0) values the main scatters seem to be the tetramer (abcd), not the dodecamer (abcd)$_3$, as suggested by Krebs et al. (45). Fig. 5C corresponds then to the tetramer shown schematically in Fig. 1C. The dissociation at pH 9.0 is not consistent with the 1/12 subunit of HbGp, which corresponds to (abcd)$_3$L$_3$ as shown in Fig. 1B and a molecular mass of 290 kDa, and according to the I(0), the main scatter in the solution is closer to the tetramer (abcd) of 68–70 kDa. It is worth mentioning that in the presence of 20 mM SDS the scattering curve at alkaline pH presents a broad peak in the q region of 0.19 Å$^{-1}$, characteristic of SDS micelle-like aggregates, similar to the way it does at pH 7.0 (Fig. 4B). It means that a high surfactant concentration does not lead to further protein dissociation at such alkaline pH.

It is interesting that fluorescence titration of HbGp at pH 9.0 with increasing SDS concentrations (data not shown) shows a steeper initial 2-fold increase up to 1 mM surfactant, and the overall curve resembles a binding isotherm; at 20 mM SDS a 5-fold increase in fluorescence is observed. It should also be remarked that increase of pH from 7.0 to 9.0 leads to oligomeric dissociation and a corresponding 3-fold fluorescence increase. These results again suggest that some dissociation occurs at low concentrations of SDS followed by denaturation at higher surfactant concentrations.

In the case of met-HbGp at pH 7.0, in the absence of SDS, the SAXS curve (Fig. 6A) is identical to that obtained for oxy-HbGp at the same pH, leading to identical $p(r)$ functions (Fig. 6C). However, in the presence of 2 mM SDS (Fig. 6B), the changes observed in the scattering profile are more pronounced as compared with those obtained for the oxy-HbGp at the same SDS concentration (Fig. 3B). The decrease in the initial intensity is greater (more than 10-fold) and the SAXS data lose completely the characteristic shoulders of extracellular hemoglobins. The $R_g$ value decreases to 65 ± 10 Å, and comparison of initial intensities suggests that, in this case, the 1/12 subunit of HbGp with molecular mass of 290 kDa (Fig. 1B) could be the main scatter. This indicates clearly that the oxidation state of the iron atoms interferes in the protein dissociation in such a way that the met-form of the macromolecule at pH 7.0, in the presence of 2 mM SDS, is more unstable as compared with the oxy-form at the same pH. The corresponding normalized $p(r)$ curve (Fig. 6C) can corroborate with the idea of 1/12 subunit dissociation; there is a major contribution of the internal distances of the scattering particle with a maximum at $r$ = 30 Å, followed by contributions of larger distances at $r$ ranging from 100 to 200 Å. Then, the $p(r)$ function exhibits a decrease achieving zero at $r$ = 300 Å.

The fluorescence titration of the met-form with increasing concentrations of SDS shows on the one hand, a steeper fluorescence increase at pH 7.0 as compared with the oxy-form and consistent with the protein dissociation at lower surfactant concentration and, on the other hand, at pH 9.0 a very small fluorescence increase upon SDS addition. These data support the findings described above in SAXS experiments.

Previous results from fluorescence technique reported in the literature (10) have shown a more extensive dissociation at alkaline pH of the met-form as compared with the oxy-form. From our results, however, the met-HbGp at pH 9.0 is characterized by a scattering curve (Fig. 7A) very similar to that observed for oxy-HbGp (Fig. 5A), leading to similar $p(r)$ function (Fig. 7B) and, consequently, to the same radius of gyration (Table I). Therefore, such a finding indicates the tetramer (abcd) as the main scatter for the met-form at pH 9.0. A surprising result is found, indeed, for the system composed of met-HbGp at pH 9.0 in the presence of 0.2 and 2 mM SDS (Fig. 7A). A different scattering curve profile is observed, displaying a big increase in the intensity for small q values, characteristic of the presence of large aggregates in solution with $R_g$ of at least 94 Å as evaluated from Guinier’s plot. Certainly, more than one species may be scattering in the surfactant-protein solution. In this case, it was not possible to perform the $p(r)$ analysis. Maybe, an equilibrium of different aggregates of tetramer, (abcd), (abcd)$_2$, (abcd)$_3$, etc., as well as aggregates of linker chains, can take place in solution. As a conclusion, for met-HbGp at pH 9.0, the presence of surfactant up to 2 mM seems to induce some kind of aggregation. At the larger SDS concentration of 20 mM these differences tend to disappear and again the SAXS (data not shown) curves are the same for both oxidation states of iron.

**DISCUSSION**

The oligomeric structure of native HbGp in solution at pH 7.0 is very similar both regarding dimensions and molecular shape to the homologous Hb of *L. terrestris*, HbLt. The dimensions obtained from $p(r)$ function and the packed beads models are consistent with those obtained from cryo-microscopy image reconstruction for HbLt. At pH 7.0 for oxy-HbGp, the surfactant produces two different effects on the protein, related to the dissociation process; initially, at 2 mM SDS the hexagonal bilayer separates into two-three smaller close-to-hexagonal structures, and then, at 20 mM SDS the dissociation into...
smaller subunits such as tetramers must take place. Of course, if a single protein in solution was simply separated into two identical subunits, the zero-angle scattering intensity would be around half of its initial intensity, and a reduction of 50% would be expected and not of 60% as observed at 2 mM surfactant. As the change of the electron contrast of the scattering macromolecule caused by SDS would be negligible at 2 mM, the 10% extra decrease of molecular mass of the single hexagonal layer with SDS binding might be explained by some further dissociation of part of the whole half of the molecule with some loss of molecular mass. Another reason for this discrepancy could be some additional dissociation of the hexagonal layers producing smaller subunits contributing to the total scattering. The extrapolated initial scattering intensity of the SDS-protein complexes at 20 mM SDS decreases 40-fold from the initial scattering in the absence of surfactant, suggesting that the molecular mass of the scattering particle shall be around 77 kDa, which corresponds approximately to the molecular mass of the tetramer (abcd, 68 kDa) assuming each monomer has a similar mass around 17 kDa. The difference of 9 kDa could well be due to the bound surfactant, which is associated with the protein. In fact, this result could be compared with data reported by Krebs et al. (45) for HbLt, where the scattering particle with $R_g$ of 37 Å and $D_{max}$ of 106 Å was associated with

**Fig. 5.** A, experimental small angle x-ray scattering curve obtained at 25 °C for 3 mg/ml oxy-HbGp in 20 mM Tris-HCl buffer at pH 9.0. B, the normalized $p(r)$, obtained from the GNOM program. C, the densely packed beads model obtained from the $p(r)$ curve with frontal view. The continuous line (in A) corresponds to the best intensity fitting obtained from the GNOM program to the experimental scattering curve.

**Fig. 6.** Experimental small angle x-ray scattering curves obtained at 25 °C in 20 mM Tris-HCl buffer at pH 7.0 of met-HbGp 3 mg/ml in: buffer solution (□ (A)), the presence of 2 mM SDS (○ (B)), the normalized $p(r)$ obtained from GNOM program for both scattering curves (C). The $p(r)$ curve for 3 mg/ml oxy-HbGp at pH 7.0 is also shown for comparison (●). Continuous lines correspond to the best intensity fitting obtained from the GNOM program to the experimental scattering curves.
the dodecameric subunit of the *L. terrestris* hemoglobin, (abcd)3. It should be mentioned that the assumption of dodecamer made by Krebs et al. (45) was not based on a determination of molecular mass from SAXS data but only on the results obtained from chromatography of the protein dissociated in the presence of 4% urea. In this context the slightly lower \( R_g \) observed in our case (practically within the experimental uncertainties) together with the estimated molecular mass from I(0) would be due to the fact that the main scatters are indeed the tetramers. The crystallographic structure for the dodecameric subunit has been resolved at 2.9-Å resolution and reported by Martin et al. (43). Based on a unit cell volume of \( 3.6 \times 10^5 \) Å\(^3\), a radius of 44 Å must be obtained. It is worth mentioning that in a pure surfactant solution, at 20 mM SDS, micelles are formed with an aggregation number close to 60–80 implying that a micelle aggregate of roughly 17–23 kDa is also contributing to the overall scattering. Therefore, SDS micelles and dissociated protein subunits (and/or subunit-SDS complexes) must contribute to the observed total scattering. In this way, the observed radius of gyration of 30 Å at 20 mM SDS (see Table I) could well represent an average value of micellar and dissociated protein subunits scattering.

It is also worth mentioning the paper of Inouye et al. (46), which describes the tetrameric assembly of protein zero myelin glycoprotein based on synchrotron SAXS experiments of a protein sample in 0.1% SDS solution (3.5 mM SDS). By means of electron density profile obtained from crystal structure it was possible to model different parts of the protein-surfactant complex. Unfortunately, the crystal structure data obtained for HbLt by Royer et al. (5) are still not available, precluding, at this stage, calculations of scattering curves for our protein to compare with the experimental data. In the paper mentioned above (46) the authors observed an intensity maximum at 0.003–0.004 Å\(^{-1}\), dependent on protein concentration due to interparticle interference. In our experiments, since the minimum \( q \) value is around 0.008 Å\(^{-1}\), this low \( q \) range was not accessible in the experiments. However, the scattering curves in the range 0.8–6.0 mg/ml are essentially identical for HbGp suggesting the absence of this interference effect. All further analysis of particle dimensions and structure performed in Ref. 46 is based on the use of correlation functions to simulate the scattering data. We believe that at this stage of our work without the knowledge of detailed electron density profiles from crystal structure the determination of the stoichiometry of surfactant binding is still not possible. This is certainly an interesting matter for future studies.

It is also of interest to consider the differences observed at 2 mM SDS between the oxy- and met-forms of HbGp. As shown in Fig. 5 the \( p(r) \) function for met-HbGp is quite different from that of met-HbGp in the absence of surfactant. Such behavior could be explained by assuming that the inner part of the \( p(r) \) is associated with the distances within each individual (abcd) monomer (in comparison with the \( p(r) \) features in the Fig. 5B) that composes the (abcd)\(_3\) structure (Fig. 1B). The distances between 100 and 200 Å should be related to the monomer–monomer inter-distances comprising the (abcd)\(_3\) triangular-like shape arrangement, while the larger distances are, of course, associated with the overall (abcd)\(_3\) structure in the incomplete hexagonal layer.

Previous studies with HbLt have shown that the cooperativity of oxygen binding is very sensitive to pH values as well as to the state of the protein, that is, as the whole intact protein in blood, extracted and purified whole protein, or the purified dodecamer, (abcd)\(_3\). At pH 6.5, the differences in Hill’s cooperativity coefficient, \( n_{50} \), are small and become important at pH > 7. At pH 7.8, where blood cooperativity is maximal, the \( n_{50} \) of the dodecamers is ~50% of the Hb and ~25% of the blood value (45). The difference in cooperativity between the dodecamer and Hb is probably due to additional interactions in the native hexagonal bilayer structure, globin-linker interactions between dodecamer and linker(s) chains, or globin-linker-globin interactions between neighboring dodecamers mediated by linker(s) chains or both. Furthermore, globin-linker-globin interactions could be either equatorial or axial within the plane of a hexagonal ring or perpendicular to it. All the hexagonal bilayer structures exhibit a relative angular displacement of ~15° between the two hexagonal layers, and Lamy et al. (1) have proposed that the relative movement of the latter may be involved in the globin-linker-globin interactions responsible for the full cooperativity in the native Hbs. The fact that the oxygenation does not lead to changes in shape of the whole Hb but the oxidation of the hemes renders the protein more susceptible to surfactant-induced dissociation is a strong evidence for changes in the subunit interactions upon oxidation of the heme iron, in agreement with our previous fluorescence studies (9, 10). It would be of great interest to monitor the effects of surfactants on the oxygen binding and cooperativity of HbGp to understand more fully all the structural changes and modifications of subunit interactions involved in protein function and described in the present SAXS studies.

**CONCLUSION**

The foregoing results allow a number of conclusions regarding both the structure of HbGp and the SDS-HbGp protein interaction in aqueous solution; the scattering data of the protein in the presence of 20 mM SDS at pH 7.0 (Fig. 2B) are similar to the data obtained for the hemoglobin at pH 9.0 in the absence of surfactant (Figs. 4A and 5A), suggesting that the mechanism of dissociation in the presence of high amount of SDS is similar to the mechanism at alkaline pH 9.0, at least as monitored by the SAXS curves; fluorescence experiments, however, suggest that care should be made in this comparison, since the 11-fold increase for 20 mM SDS, pH 7.0, is greater than the 3-fold increase observed at pH 9.0, and this should be due to some protein denaturation at high surfactant besides
associated with a significant number of surfactant molecules where the oxidation state of the iron atoms contained in the heme oxy-form under the influence of 2 mM surfactant, and apparently the 1/12 subunit is the main scatter in solution; this dissociation of the met-form is also observed from fluorescence data, although a straightforward comparison is difficult due to the different concentrations used for the two techniques.

Dissociation at alkaline pH 9.0 for oxy-HbGp seems to give, as the main scattering particle, the tetramer. In the case of met-HbGp the SAXS data in the absence of surfactant are similar to the data for oxy-HbGp at pH 9.0, while in the presence of SDS up to 2 mM concentration some kind of aggregation seems to be induced, as reflected in the scattering intensity at low q. The polydispersity of the solution precludes an accurate determination of p(r) from the GNOM program. At 20 mM SDS the scattering for oxy- and met-forms of HbGp is quite similar. Unfortunately, it is not possible to define precisely the main scatters at 20 mM surfactant, since the obtained p(r) distribution function carries information about the possible protein subunit-free micelles and/or those made up by association with the subunits. Besides that, the presence of free micelles in this condition cannot be ruled out, making the observed scattering an average of that of several particles in solution.

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Small Angle X-ray Scattering (SAXS) Study of the Extracellular Hemoglobin of *Glossoscolex paulistus*: EFFECT OF pH, IRON OXIDATION STATE, AND INTERACTION WITH ANIONIC SDS SURFACTANT

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