Band 3 catalyzes sickle hemoglobin polymerization

Maria A. Rotter [a,1], Haiyan Chu [b], Philip S. Low [b], Frank A. Ferrone [a,*]

[a] Department of Physics, Drexel University, Philadelphia, PA 19104, United States
[b] Department of Chemistry, Purdue University, West Lafayette, IN 47906-1393, United States

ARTICLE INFO

ABSTRACT

We have measured homogeneous and heterogeneous nucleation rates of sickle hemoglobin (HbS) in the presence of a strongly binding deletion mutant of the cytoplasmic domain of band 3 (cdb3), a membrane protein known to form dimers and to bind 2 HbS molecules to such a dimer, and we find that it accelerated both rates by a factor of 2. A weakly binding mutant, in contrast showed no impact on nucleation rates, contrary to naïve expectations of a slight enhancement based on the molecular crowding of the solution by the mutant. We find we can explain these phenomena by a model of HbS–cdb3 interaction in which the strong binding mutant, by stabilizing an HbS dimer, catalyzes the nucleation process, while the weak mutant binds only 1 HbS molecule, effectively inactivating it and thereby compensating for the crowding of the solution by the cdb3. The catalytic behavior we observe could play a role in intracellular processes.

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1. Introduction

Sickle cell disease arises from the polymerization of sickle hemoglobin (HbS) molecules inside the red cell, and is one of the oldest of a large and growing class of diseases that derive from a pathological assembly process. The polymerization of HbS is made possible by a mutation of β6 from Glu to Val, and the resulting polymers rigidify the red cell. The polymerization process begins with homogeneous nucleation during which an unstable cluster or nucleus forms in solution and initiates the first polymer in a given region. The polymer that appears from the homogeneous nucleus then permits the nucleation of new polymers on its surface in a heterogeneous nucleation pathway that generates arrays of attached polymers called domains [1]. Since all polymers in a domain are generated because of one homogeneous nucleation event, domains appear after a variable delay corresponding to the random waiting time before the first nucleus is formed [2]. When many domains are seen simultaneously, as in bulk samples, the randomness disappears owing to the averaging effect of large numbers. Exponential growth of the polymer mass gives a further apparent lag, known as the "delay time", though as the sensitivity of the probe increases, it becomes possible to detect polymers at earlier and earlier times. This contrasts with the stochastic delay, which is the random waiting time before the first nucleus (and then polymer) appears, and for which there is no evidence of the passage of time. Both delays are physiologically significant, since, if sickling is postponed until the venous circulation where the cells are not constrained, the cells can reach the lungs where reoxygenation will induce depolymerization.

A recurring question has been whether the cell membrane and its many constituent proteins have any effect on polymerization [3–8]. Because most polymers are formed by heterogeneous nucleation, the answer to this question is not easy to obtain. Yet in a red cell the effects can be profound, since the stochastic delay and its correspondingly protective effect would be eliminated if membrane proteins instigated nucleation. Recently we have shown that membrane fragments from sickle red cells can assist nucleation, while membranes taken from AA cells appear to have no such effect [9]. This immediately suggests that membrane damage in sickle patients leads to further sickling, and perhaps further damage. The mechanism for the acceleration of nucleation has not been discovered.

A major membrane component that is known to bind to Hb is band 3. The cytoplasmic domain of band 3 (cdb3) binds Hb by inserting its acidic NH_2-terminus between the beta chains of Hb in the same cleft occupied by BPG or IHP [10]. Cdb3 is also strongly associated into dimers. Site directed mutants have been created that strongly bind Hb to each cdb3 dimer in contrast to wild type cdb3 that binds the second Hb only weakly. Based on the assumption that, like wild type cdb3, the mutant binds 2 Hb molecules, we hypothesized that if the Hb molecules bound to cdb3 themselves bond together as a dimer, this could enhance nucleation by their prevalence over natural dimers.

To investigate this relatively simple idea requires that certain corrections for solution crowding be taken into account. All reactions occurring in media in which a considerable fraction of space is occupied are sensitive to nonideal or "crowding" effects which favor associated states as a means to lower the free energy of the system

[11]. This means that even the introduction of an inert molecule, in sufficient quantity, can favor association reactions, thereby requiring careful analysis to determine whether the added molecule plays an active (i.e. catalytic) or passive (crowding) role. Fortunately, the description of such nonideality has been developed to the point where it can be used confidently in the analysis of the reactions.

In this paper we examine the effect of strong and of weak binding cdb3 mutants on the nucleation of HbS. We wished to determine whether band 3 might be capable of affecting nucleation and if so, then to develop an explanation for any such effects consistent with known Hb and band 3 behavior.

2. Materials and methods

Sickle hemoglobin (HbS) was prepared by column chromatography, using standard procedures. Samples of HbS were exchanged into 50 mM, pH 6.8 HEPES buffer and concentrated to approximately 40 g/dl by Centricon Centrifugal Filter Units (Millipore). Two mutants of cdb3 were prepared as described elsewhere [12]: a high affinity mutant that lacks residues 29–52 (referred to here as cdb3+) and low affinity mutant that lacks residues 12–23 (referred to here as cdb3−). The high affinity mutant increases the p50 (50% saturation oxygen pressure) of oxygen binding from 12 mm Hg to 175 mm Hg in an equimolar solution of cdb3+ and Hb. In contrast, the low affinity mutant exerts no effect on Hb-O_2 affinity. The initial concentrations of cdb3+ and cdb3− were 46.8 mg/ml and 42.2 mg/ml, respectively.

Highly concentrated HbS (40 g/dl) was placed into a CO-saturated glove box. Sodium dithionite was added to the HbS solution to achieve a final concentration of 50 mM. 20% of cdb3 protein solution was added to the HbS solution to yield a 98% HbS/2% cdb3 mixture. The mixtures were then diluted with buffer to the concentrations shown in Fig. 1. HbS concentration was determined by diluting the hemo-globin solution 3000 times using micropipettes and measuring the Soret absorption spectrum of the dilute sample in a cuvette with a 1 cm path length.

Kinetics of polymerization of HbS were measured using laser photolysis of HbCO [13]. Forward scattered light permitted us to follow the kinetics, which grow exponentially as $A\exp(Bt)$ following a random delay corresponding the first nucleus being formed. The exponential rate B is dominated by the rate of heterogeneous nucleation [1]. By separating the laser beam into a large number of spots (~100), we could employ the stochastic variation of onset times to determine the rate of homogeneous nucleation [14], designated f_o.

3. Results

HbS samples were prepared in pH 6.8 HEPES buffer with concentrations ranging from 3.9 to 5.1 mM (25 to 33 g/dl). A mutant cdb3 with residues 12–23 deleted was prepared, which exhibits only about 20% of the affinity for Hb (in the presence of BPG) as wild type cdb3, and is thus designated cdb3−. This mutant was added to yield a final concentration of 0.226 mM. High affinity cdb3 (cdb3+) was prepared in which residues 29–52 are deleted and which displaces BPG and binds Hb about twice as strongly as wild type cdb3 [12] This was added to a final concentration of 0.232 mM. HbS samples were also examined in the same buffer in the absence of any cdb3.

Addition of cdb3 led to no qualitative differences in the appearance of polymerization compared to samples without added cdb3: polymerization exhibited exponential growth of light scattering following stochastic delays. High concentration dependence of both homogeneous nucleation rate (f_o) and exponential growth rate (B) was also observed.

The most direct method of comparison would have been to prepare solutions of identical concentrations with no cdb3 as well as high and low affinity cdb3 variants. Because of the small quantities of Hb used and the extraordinary sensitivity of the rates to concentration [15], it is difficult to prepare samples with concentrations that are sufficiently close for direct comparison, even though the same range of data can be spanned. To compare the different samples we thus used the following empirical interpolation procedure. We first fit the data for HbS polymerization without band 3 to a second order polynomial, viz., $y = a_o + a_1x + a_2x^2$ where $x = \log c$ and y is $\log f_o$. We then plotted the difference of $\log f_o$ for HbS with cdb3 relative to the function determined from the polynomial, including the data originally used to generate the fit. This has the effect of suppressing the large underlying concentration dependence so as to observe differences as the various band 3 components are added. This procedure does not require the fit to be highly accurate, but simply acts as a way to adjust the display for a more intuitive and direct understanding of the data. The results are shown in Fig. 1, in which the triangles are for the HbS without cdb3, the empty circles refer to the HbS with cdb3− and the filled circles describe the HbS with cdb3+. It is evident that the filled circles are systematically above the empty symbols (pure HbS and HbS with cdb3−), which themselves are quite similar to one another. The horizontal lines show the average deviation of each data set from the reference polynomial. Thus the horizontal solid line at zero is, by construction, the average of the triangles (pure HbS). The dotted line, almost at the axis, is the average of the open circles (HbS + cdb3−), and the long dashed line, 0.30 units above the axis, is the average of the filled circles (HbS + cdb3+). Without the use of this reference polynomial, the data vary by 10^6 over this range (from −10 to −4 on the log scale). With such variation, the 0.30 change in $\log f_o$ observed here, which is a factor of 2 in nucleation rates, is more difficult to see clearly in the absence of the referencing system employed.

A similar procedure was employed for the analysis of the log of the exponential growth rate, viz. $\log B$. This is shown in Fig. 2. Once again
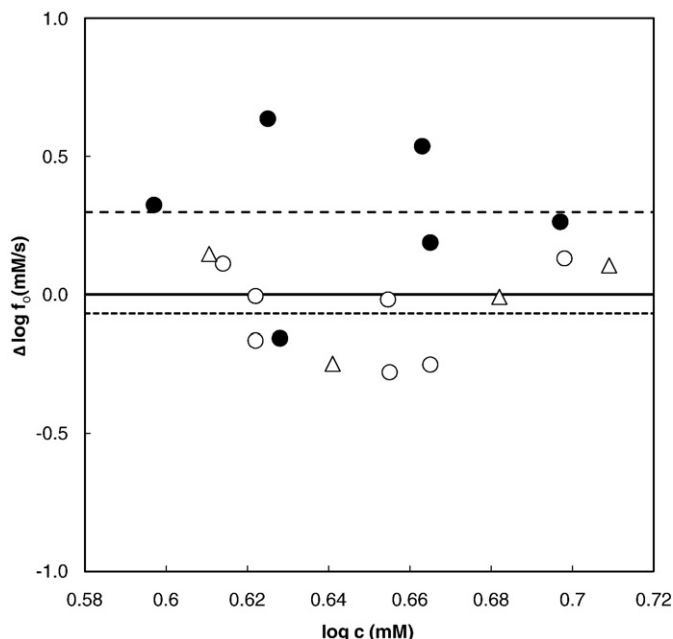
Fig. 1. Log of homogeneous nucleation rate, f_o, after subtracting a second order polynomial fit to HbS without cdb3, plotted as a function of log of initial HbS concentration. The polynomial used was $\log f_o = -148.3 + 379 \log c - 249 (\log c)^2$. Filled circles show Hb with the strong binding variant (cdb3+), while open circles show the weak binding variant (cdb3−). Open triangles show HbS in the same buffer without any cdb3, to which the polynomial was fit. The solid line is the fit to HbS+, the long dashed line is the average of the deviations HbS with cdb3+ while the dotted line is the average of the deviations of HbS with cdb3+ from the polynomial. It is clear that the HbS with cdb3+ (filled symbols) has a higher nucleation rate than the other data (open symbols).
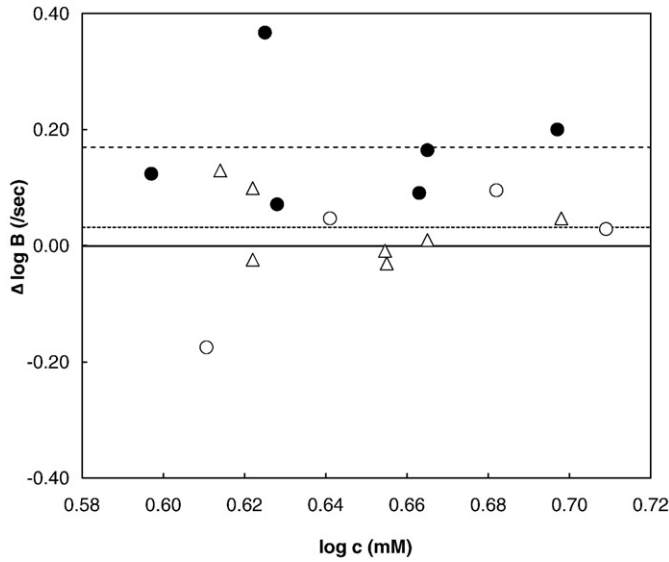
Fig. 2. Log of exponential growth rate B after subtracting a second order polynomial fit to HbS without cdb3, plotted as a function of log of initial HbS concentration. B is dominated by the square root of the heterogeneous nucleation rate, and changes only slightly due to the homogeneous nucleation rate. The polynomial used was $\log B = -27 + 68 \log c - 39$ $(\log c)^2$. Filled circles show Hb with the strong binding variant (cdb3+), while open circles show the weak binding variant (cdb3−). Open triangles show HbS in the same buffer without any cdb3, to which the polynomial was fit. The solid line is the fit to HbS+, the long dashed line is the average of the deviations HbS with cdb3+ while the dotted line is the average of the deviations of HbS with cdb3+ from the polynomial. $\log B$ is clearly greater for the filled symbols.

the HbS data and that with cdb3− added are very similar, while the exponential growth rate for HbS with cdb3+ (filled circles) is distinctly faster. The dashed line average is 0.17 above the reference polynomial. Because it is related to the square root of the heterogeneous nucleation rate, B would be expected to show less variation than the homogeneous nucleation rate if both had been equally affected. This appears to be the case in that the change in $\log f_o$ (0.30) is close to twice the change in $\log B$ (0.17).

4. Discussion

The first major result of this study is that HbS nucleation can be enhanced by the mutant of band 3 with elevated affinity for Hb [12]. From the enhancement in nucleation rates observed, one might infer that cdb3+ binds two Hb molecules in sufficient proximity for the two hemoglobins to bind each other, thereby facilitating formation of a stable dimer and augmenting nucleation. (Throughout this discussion, the term Hb dimer refers to two Hb molecules, each of molecular weight 64 kD.) Support for such a view is found from the binding of Hb to wild type cdb3. Hb binding to cdb3 is anti-cooperative, with the second molecule binding almost 3 fold less strongly than the first [16]. This is readily rationalized as the result of the Hb molecules being close enough to provide steric inhibition. It might seem counterintuitive that catalysis could result when there is a penalty for binding the second Hb molecule to a cdb3 dimer. However, this is an expected consequence of the nucleation process. Nucleation entails initially unfavorable reaction steps that eventually become favorable, and thus dimer formation is always one of the unfavorable steps (so long as the size of the nucleus is larger than 2). Therefore, even if the second molecule of the dimer is not bound as strongly as the first, the simple existence of a stabilized dimer is a substantial advantage compared to the usual unstable dimer. The fact that both homogeneous and heterogeneous nucleation are affected equally, another important finding, is also an expected outcome from such

an explanation, since stabilization of nuclei (via the dimers) can happen the same way for each pathway.

The rates are enhanced because the nuclei are more plentiful. Thus to rationalize the result it is necessary to consider the formation of nuclei with and without cdb3. The nucleation reaction is formally written as the joining of n molecules of Hb, thus

$$n Hb \overset{K_n}{\rightleftharpoons} Hb_n. \tag{I}$$

The concentration of nuclei, in an ideal reaction, is given by $c_n = K_n c^n$. However in a concentrated solution, activity coefficients, γ, must be considered, and the reaction becomes $\gamma_n c_n = K_n (\gamma c)^n$ where the convention used here is that unsubscripted γ refers to monomers. All activity coefficients are a function of the solution concentration, but to keep the notation slightly simpler this concentration dependence will not be explicitly written as $\gamma(c)$ even though it is implied whenever activity coefficients appear. In general the activity coefficient is a function of the concentration of *all* species, but since the Hb monomers are in such excess, they will be the only significant contributors. The actual computation of activity coefficients will be described later.

For analysis, the nucleation process can be conceptually separated into dimer attachment to an aggregate of size $n-2$. This reaction is designated with the equilibrium constant K_{+2}.

$$(n-2)Hb + Hb_2 \overset{K_{+2}}{\rightleftharpoons} Hb_n \tag{II}$$
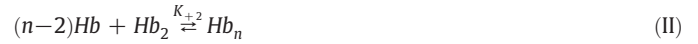
We can describe the equilibrium of reaction (II) as

$$\gamma_n c_n = K_{+2}(\gamma c)^{n-2} \gamma_2 c_2. \tag{1}$$

Then we wish to consider the reaction in which the Hb dimers that are part of the nucleus are replaced by Hb dimers attached to band 3. Band 3 dimers will be designated B_2 and the complex will be designated $B_2 Hb_2$.

$$(n-2)Hb + B_2 Hb_2 \overset{K'_{+2}}{\rightleftharpoons} B_2 Hb_n \tag{III}$$

The binding constant now is designated with a prime. If r is defined as the ratio of $[B_2 Hb_n]$ to $[Hb_n]$, the twofold enhancement in nucleation rate arises because $r = 2$, i.e. there are twice as many nuclei with cdb3+ than without it. Reaction (III) is described by

$$\gamma'_n c'_n = K'_{+2}(\gamma c)^{n-2} \gamma'_2 c'_2 \tag{2}$$

where the primes indicate that a band 3 dimer is bound to a nucleus or a Hb dimer.

The final reaction to complete the sequence is the binding of HbS to band 3, viz.

$$2Hb + B_2 \overset{K_b}{\rightleftharpoons} B_2 Hb_2 \tag{IV}$$

The binding of dimers of HbS to the cdb3 dimers, which have concentration b_2 and activity coefficient γ_b can be written as

$$\gamma'_2 c'_2 = K_b \gamma_b b_2 \gamma_2 c_2 \tag{3}$$

and finally the concentration of Hb dimers is described by

$$\gamma_2 c_2 = K_2 (\gamma c)^2. \tag{4}$$

Eqs. (3) and (4) describe reaction (IV) above.

To combine these relations, because the cdb3+ dimers are so strongly attached to Hb it is necessary to write the equation in terms

of the initial concentration of cdb3 dimers, b_2^o. Bound cdb3 plus free gives the total (initial) concentration, thus,

$$K_b(\gamma_b \gamma_2 / \gamma_2')c_2 b_2 + b_2 = b_2^o. \tag{5}$$

From this

$$b_2 = b_2^o / (1 + K_b(\gamma_b \gamma_2 / \gamma_2')c_2) \approx b_2^o / K_b(\gamma_b \gamma_2 / \gamma_2')c_2 \tag{6}$$

since the strength of association insures that there is almost no free cdb3+ and thus that $K_b(\gamma_b \gamma_2/\gamma_2') c_2 \gg 1$. Therefore r, the ratio of the rates, which is equal to the ratio of concentration of nuclei with cdb3 to without cdb3, is given by

$$r = (K_{+2}'(\gamma c)^{n-2}\gamma_2' c_2' / \gamma_n') / (K_{+2}(\gamma c)^{n-2}\gamma_2 c_2 / \gamma_n) \tag{7}$$
$$= (K_{+2}' / K_{+2})\gamma_2'(\gamma_n / \gamma_n')(b_2^o / \gamma_2 c_2)$$

in which last equality comes from substitution of previous equations and cancellation of terms.

To simplify calculation of the activity coefficients we can make the approximation that, since the cdb3+ is close in size to Hb, activity coefficients which include the cdb3+ dimer are equivalent to activity coefficients augmented by a dimer of Hb. Therefore, $\gamma_2' \approx \gamma_4$ and $\gamma_n' \approx \gamma_{n+2}$, giving

$$r = (K_{+2}' / K_{+2})\gamma_4(\gamma_n / \gamma_{n+2})(b_2^o / K_2(\gamma c)^2). \tag{8}$$

These activity coefficients are computed from scaled particle theory, viz.:

$$\ln \gamma_n = -\ln(1-\phi) + \frac{3\phi}{1-\phi}\left(\frac{n}{\rho}\right)^{1/3} + \frac{3\phi + \frac{9}{2}\phi^2}{(1-\phi)^2}\left(\frac{n}{\rho}\right)^{2/3} \tag{9}$$
$$+ \frac{\phi(1 + \phi + \phi^2)}{(1-\phi)^3}\left(\frac{n}{\rho}\right)$$

in which ϕ is the volume fraction occupied, i.e. $\phi = vc$ where v is the specific volume of the Hb (0.79 g/cm^3) and ρ is the relative density of the aggregate relative to the monomer (0.5) [17]. The above equation can be evaluated by recourse to the theory of nucleation that has been successful in describing Hb polymerization [1,18–20]. That allows n to be calculated, as well as the activity of the dimer ($\gamma_2 c_2$). For [Hb] = 4.5 mM, it is found that $n = 6.2$, and the activity of the normal dimer, given by $K_2(\gamma c)^2$ is 1.0 μM. (The concentration of the dimer, which is the activity divided by the activity coefficient, would be 8.6 pM.) The activity coefficients are found to be: ln $\gamma_4 = 11.69$, ln $\gamma_n = 16.2$ and ln $\gamma_{n+2} = 20.1$. The concentration of cdb3+ is 0.12 mM. The only unknown in this equation is the ratio of attachment equilibria, (K_{+2}'/K_{+2}). By solving Eq. (8), this ratio is then found to be 6.6×10^{-6}, i.e., given an HbS dimer, it is 6.6×10^{-6} times less likely to grow into a nucleus if that dimer is attached to cdb3+ than if the nucleus is a pure HbS dimer. The reason for the overall enhancement of nucleation rates is that, the above hindrance notwithstanding, it is so much more likely to *have* a dimer with cdb3+ attached (0.12 mM) than to have a pure dimer (8.6 pM). Another way to express the reluctance for the dimer to form the nucleus is by the associated free energy, RT ln (K_{+2}'/K_{+2}). This effectively represents the penalty of having a dimer of cdb3+ attached to a nucleus, and is 7.1 kcal/mol. Two things should be noted. One is that the calculations are based on model parameters derived in the presence of phosphate buffer at pH 7.35, whereas the present data is collected in HEPES buffer, pH 6.8. However, the solubility appears to be asymptotic at low pH, so we do not expect much difference [21]. Secondly, in the calculation of aggregates, the assumptions that generate dimer concentrations are at their weakest. Changes could alter some of the specific parameters, but would not change the overall point of the analysis, which is that significant

enhancement is offset by a significant penalty, to the point where the net effect is modest. Or put another way, were it not for the penalty the cdb3+ attachment imposes, the nucleation rate would have been four orders of magnitude faster than normal.

The above discussion also suggests why cdb3– has no measurable effect on polymerization as seen in Figs. 1 and 2. While it might have been expected that a weakly interaction additive would simply leave the normal nucleation rates unchanged, the presence of the 0.2 mM protein ought to have a small but observable enhancement due to molecular crowding. The preceding analysis explains why essentially an exact cancellation is expected. The powerful inhibitory effect of having cdb3 bound is not compensated by the presence of dimers, since the cdb3– does not bind a second Hb strongly. On the contrary, the crowding agent, as it binds to a population of HbS, inactivates it. Thus, the crowding, which promotes polymerization, is offset by the direct, presumably steric effect of bound cdb3–.

Finally, an interesting issue addressed by this work is whether larger assemblies could act as templates for larger HbS oligomers. Such aggregates of band 3 could be the consequence of HbS oxidation or membrane damage in the course of polymer formation, and might also enhance nucleation further. There is evidence for clustering of band 3 in sickle cell membranes [22] as well as membranes of dense [23,24] or senescent cells [24]. The effect of such oligomers is magnified by the infrequency of finding the equivalent Hb oligomers, due to the barrier that accounts for nucleation. For example, Hb tetramers (i.e. 4 Hb molecules) would only be expected to have a concentration of 78.5 fM concentration (with 9.4 nM tetramer activity). However, a critical issue is the modification of the ratio (K_{+2}'/K_{+2}) for a larger oligomer. For example, if one assumed that the repulsion is energetically additive, then for a tetramer it is straightforward to show that the repulsion would substantially outweigh the gains achieved because of the natural scarcity of tetramers. On the other hand, were there no repulsion, aggregate forms would have a far greater enhancement than dimers do.

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