# EVIDENCE FROM SEDIMENTATION VELOCITY FOR DISSOCIATION EQUILIBRIUM IN HEMERYTHRIN

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Hemerythrin, a non-heme iron protein of sipunculids, interacts with iron coordinating ligands (Keresztes-Nagy and Klotz, 1965) and sulfhydryl reagents (Keresztes-Nagy and Klotz, 1963). These reactions are easily detected, since when an external ligand is bound by the iron the visible spectrum of the protein is changed, and when the -SH group is blocked the octameric protein dissociates into subunits. The protein -SH groups do not readily react with organic mercurials, or N-ethyl maleimide, in the absence of iron-coordinating ligands. However, in the presence of ions such as azide or thiocyanate, the reaction proceeds readily (Keresztes-Nagy and Klotz, 1965). This behavior cannot be due to a direct interaction between the iron and -SH groups since the reaction with sulfhydryl-blocking reagents is not

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accompanied by a change in the protein spectrum.

The enhanced reactivity of the -SH groups when the environment about the irons is changed is an example of cooperative interactions; a reaction at one site of the protein affects the activity of a second site. A molecular interpretation of the cooperative interaction in hemerythrin has been proposed (Keresztes-Nagy and Klotz, 1965). The model described is based on three assumptions: (1) the octameric protein is in equilibrium with a small amount of monomer; (2) the monomer has a greater affinity for iron-coordinating anions than does the octamer; and (3) the -SH group in the monomer is more reactive than the -SH group in the octamer. These assumptions suffice to explain the cooperative interaction: the binding of an ion such as thiocyanate to the protein would shift the dissociation equilibrium towards the monomer form of the protein, and would, thus, enhance the reactivity of the -SH groups.

This model is not, however, the only one that can be proposed. Binding of the ligand to the octamer might generate a conformational rearrangement without dissociation of the protein. Such a rearrangement could be responsible for the increased -SH reactivity. We have searched, therefore, for experiments which might provide evidence in favor of one of these alternatives. In particular, we have attempted to design experiments to test the three assumptions of the first model.

By hybridizing native and succinylated hemerythrin (Keresztes-Nagy et al., 1965), we have shown previously that the octamer is in equilibrium with a smaller-sized subunit. In this communication we present evidence for

an octamer-monomer equilibrium.

For any oligomer-monomer equilibrium the relative amounts of the different forms of the protein will depend on the concentration. At high concentrations the protein will exist predominantly as the oligomer. With increasing dilution the fraction in the monomer form will increase until at low concentrations monomer will be the predominant species. This variation in the percentage of monomer will be reflected in the sedimentation behavior of the protein (Gilbert, 1955). At high concentrations the protein will move in a gravitational field as a single boundary with a sedimentation coefficient characteristic of the oligomer. As the protein is diluted, and the relative amount of monomer increases, either the sedimentation coefficient of the single boundary should decrease, or a second slower moving boundary should appear.

The sedimentation of hemerythrin has been measured previously with schlieren optics at concentrations from 1.5 down to 0.1% (Klotz and Keresztes-Nagy, 1963). Over this concentration range there was no indication of protein dissociation. Using the split-beam photoelectric scanning absorption optical system (Schachman et al., 1962; Lamers et al., 1963), we have now been able to measure the sedimentation of hemerythrin down to 0.0025% protein. At the lowest concentration the protein appears to exist as a monomer.

## Experimental Methods

Methemerythrin was prepared by previously-described procedures (Klotz et al., 1957) from the coelomic fluid of the marine worm Golfingia gouldii (also known as Phascolosoma

gouldii). The protein concentration was determined from the optical density at 355mμ based on an extinction coefficient of 3220 ($cm^{-1}$ liter $mole^{-1}$ iron) (Keresztes-Nagy and Klotz, 1965). Methemerythrin sedimentation was determined with the Spinco Model E Analytical Ultracentrifuge equipped with a split-beam photoelectric scanning absorption optical system (Schachman et al., 1962; Lamers et al., 1963). The protein was sedimented at 59,780 rpm in one sector of a 12-mm double sector cell, the second sector containing solvent. The concentration was varied from 0.025 to 4 mg/ml; at the high concentration the methemerythrin gradient was measured with 400 mμ wave-length light, and at the lower concentrations with 280mμ light.

## Results

The results from three runs, as obtained directly from the scanner, are shown in Figure 1. The recordings show a plot of the optical density (a measure of the protein concentration) versus distance in the cell. Methemerythrin dissolved in 0.100 M potassium thiocyanate, 0.02 M sodium acetate, pH 6.8, at 0.4% concentration sediments as a single boundary with an uncorrected sedimentation coefficient of 7.0S. The sedimentation coefficient of the octamer has been reported previously as 6.75S (Klotz and Keresztes-Nagy, 1963). At 0.0025% the 7S boundary is completely replaced by a boundary with a sedimentation coefficient of 2.0S. This is close to the 1.95S previously found for the monomer (Klotz and Keresztes-Nagy, 1963). At intermediate concentrations both the fast and slow boundaries are seen. These results show directly that the octamer and monomer are in equilibrium.

The formation of two boundaries is to be expected

when the oligomer-monomer equilibrium is slow in comparison with the time of the experiment; but two boundaries may also be found when the equilibrium is rapid (Gilbert, 1955). In the former case the fast and slow boundaries are composed of oligomer and monomer respectively. Furthermore, the sedimentation coefficients of the two components should remain constant as the protein is diluted. Also, the magnitudes of the two boundaries directly reflect the concentrations of the monomer and oligomer at the equilibrium established before the beginning of the sedimentation experiment.

If, on the other hand, the monomer-oligomer equilibrium is rapid, the slow boundary is composed of monomer, but the fast boundary is formed from monomer and oligomer. It has been shown (Gilbert, 1955) that, in this case, the two fronts will not separate completely, the sedimentation coefficient of the fast boundary will decrease as the protein concentration decreases, and the protein concentration of the slow boundary will remain constant as the protein is diluted.

Thus, one may distinguish between slow and rapid equilibria, by looking for complete separation of the two boundaries, measuring the concentration of protein in the slow boundary, and determining the sedimentation coefficient of the fast boundary.

Methemerythrin was sedimented at various concentrations in each of two solutions, 0.1M KCNS and 0.1M Tris. In Table I are given the percentages, and concentrations of protein in the slow boundary, as well as the sedimentation coefficient of the fast boundary, at each concentration of

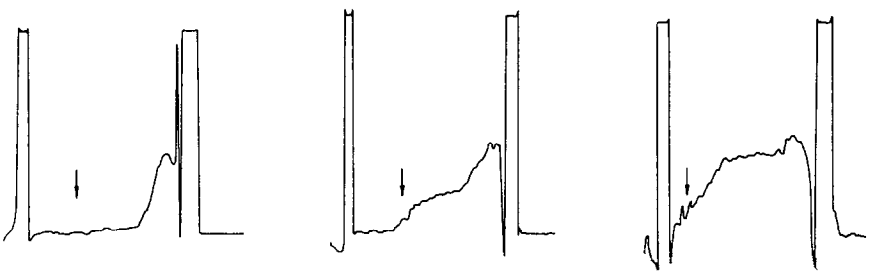

Figure 1 Dissociation of Methemerythrin

Methemerythrin was sedimented in 0.100 M KCNS, 0.02 M $CH_3COONa$, pH 6.8, as described in the text. The traces were obtained 80 minutes after the rotor had reached speed.

Left-0.40% protein
Center-0.013% protein
Right-0.0025% protein

The arrows mark the position of the solution meniscus.

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hemerythrin. The data show that the concentration of the slow component was not constant, and that the sedimentation coefficient of the fast component was constant over the range of protein concentrations tested. In addition, the derivative of the hemerythrin concentration curve (not shown here) revealed that the two boundaries were well separated from one another. These results thus indicate that the time required to establish the monomer-octamer equilibrium is long in comparison with the time of the sedimentation experiment.

The magnitudes of the optical densities of the two components are, therefore, a reflection of the octamer and monomer concentrations. At each concentration of hemerythrin more monomer was present in the KCNS solution than in the Tris buffer (Table I).

TABLE I

Dissociation of Methemerythrin

| Protein conc. (M) x $10^4$ | Percent of Slow Component | | Concentration of Slow Component (M) x $10^6$ | | $s_{20}$ of Fast Component | |
|---|---|---|---|---|---|---|
| | Tris | KCNS | Tris | KCNS | Tris | KCNS |
| 3.02 | 0 | 4 | --- | 12 | 6.7 | 7.0 |
| 1.51 | --- | 4 | --- | 6.0 | --- | 7.2 |
| .302 | 14 | 15 | 4.2 | 4.5 | 6.8 | 6.8 |
| .101 | 19 | 33 | 1.9 | 3.3 | 6.6 | 7.0 |
| .0503 | 22 | --- | 1.1 | --- | 6.7 | --- |
| .0378 | --- | 85 | --- | 3.2 | --- | 7.0 |
| .0189 | 42 | 100 | 0.79 | 1.9 | 6.5 | --- |

Sedimentation was followed as described in the text in 0.100 M KCNS, 0.02 M sodium acetate pH 6.8 at 21°C, or in 0.100 M Tris (acetate), pH 7.0 at 22°C. The percentage of the slow peak was determined from the ratio of the optical density at the plateau of the slow boundary to the total optical density measured shortly after the rotor had reached speed, 59,780 rpm. The sedimentation coefficient of the fast component was corrected to 20°C, but was not corrected for the buffer concentration.

Thus, thiocyanate promotes the dissociation of methemerythrin. Since thiocyanate is bound to the iron of the protein, whereas Tris is not (Keresztes-Nagy and Klotz, 1965), these results are in accord with the assump-

tion that the monomer binds thiocyanate more strongly than does the octamer. Under these conditions the presence of the ligand would shift the dissociation equilibrium towards the monomer, as is observed.

## Summary

Methemerythrin dissociates on dilution as shown in the ultracentrifuge with a scanner attachment. This is direct evidence for an equilibrium between the octameric protein and its subunits. Thiocyanate, which is bound to the iron of the protein, appears to increase the degree of dissociation. This behavior provides an explanation of cooperative interactions in methemerythrin.

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