SPECIFIC ANION BINDING EFFECTS ON THE REACTIVE SITES OF HEMERYTHRIN

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Hemerythrin, the non-heme iron-containing oxygen carrier of sipunculids, is composed of eight essentially identical subunits, (Keresztes-Nagy and Klotz, 1963) each of which contains two iron atoms. The octameric protein may be dissociated into subunits, each of which contains one cysteine residue (Groskopf et al., 1966), by mercaptan-blocking reagents.

It has been shown previously (Keresztes-Nagy and Klotz, 1965) that the Fe sites form complexes with a variety of anionic ligands (e.g. N_3^-, SCN^-, Cl^-), and that this coordination enhances the reactivity of the protein sulfhydryl sites as judged by the rate of dissociation of octamer into monomers.

We have now found that certain specific anions are bound to hemerythrin, at a site different from the iron locus, and produce a pronounced effect on the behavior of both iron and sulfhydryl groups.

Behavior at Iron Site

The effect at the iron locus has been evident from two types of experiments, one involving equilibria, the other rates. Typical coordination equilibria of hemerythrin may be represented by the equation

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$$[HrFe^{III}(H_2O)_m]_8 + 8nL^- = [HrFe_2{}^{III}(L)_n]^{8n^-} + 8m\ H_2O \qquad (1)$$

where L is the iron ligand (e.g. OH^-, N_3^-, NCS^-). The formation of the ligand complex creates a net negative charge at the iron locus. Binding of a non-ligand near the iron should tend to make the Fe-ligand complex less stable, thermodynamically, through electrostatic effects, and thus will drive the equilibrium in equation (1) towards the aquo side. It should also retard the rate of formation of the Fe-ligand form. Both of these effects have been observed.

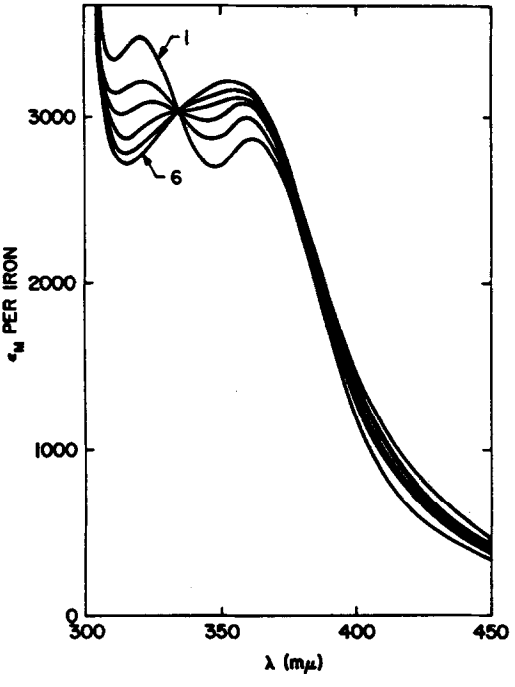

Figure 1. Absorption spectra showing conversion of met-hydroxohemerythrin to met-aquohemerythrin in the presence of sodium perchlorate at 5°C.

1. pH 8.62, 0.00 M $NaClO_4$
2. pH 8.62, 3.75×10^{-4} M $NaClO_4$
3. pH 8.62, 1.00×10^{-3} M $NaClO_4$
4. pH 8.62, 3.75×10^{-3} M $NaClO_4$
5. pH 8.62, 2.50×10^{-2} M $NaClO_4$
6. pH 6.36, 0.00 M $NaClO_4$

Non-ligand anion binding was detected by adding non-coordinating anions to a solution of met-hydroxohemerythrin $[HrFe_2^{III}(OH^-)_n]_s$, at pH 8.5. The magnitude of the spectral change (see Figure 1) towards met-aquohemerythrin, $[HrFe_2^{III}(H_2O)_m]_s$, then indicated the magnitude of non-ligand anion binding present. Using this as a criterion for anion binding we find that strong binding occurs with perchlorate and with nitrate ions, weak binding with phosphate ion and no binding with sulphate, dodecylsulphate, acetate or trichloroacetate ions. The non-ligand anion binding site appears, therefore, to be specific for certain small anions.

To ensure that the observed effects were not due to coordination to the iron, the circular dichroic spectra of these solu-

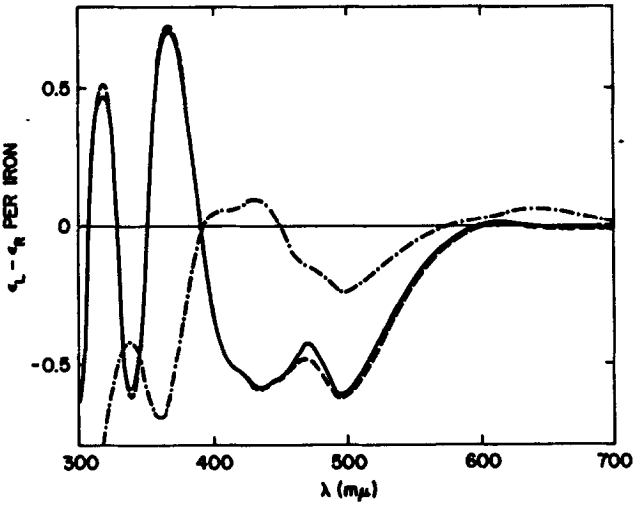

Figure 2. Circular dichroic spectra of met-aquohemerythrin and met-hydroxohemerythrin mixtures.

————pH 7.0, 87% met-aquo

——·——pH 8.5, 94% met-hydroxo

---------pH 8.5, 0.091 M $NaClO_4$, 93% met-aquo

Spectra recorded on Durrum-Jasco Recording Spectropolarimeter at 25°C in 0.1 M tris buffer adjusted to pH with solid cacodylic acid.

tions were measured and compared with that of met-aquohemerythrin at pH 7.0. These are given in Figure 2 where perchlorate is the added ion. The presence of about 0.1 M sodium perchlorate alters the circular dichroic spectrum from that of met-hydroxohemerythrin to one which is essentially identical to that of met-aquohemerythrin. Since circular dichroism is particularly sensitive to changes at the iron, it is clear that the species formed in the presence of perchlorate is met-aquohemerythrin, and not an iron-perchlorate coordinational complex.

Quantitative studies have been carried out on the effect of perchlorate and of nitrate ion binding on the aquo-hydroxohemerythrin (see Figure 1) equilibrium. For a protein concentration of 3×10^{-4} M in iron, the following pK values have been observed: 0.00 M $NaClO_4$, pK 7.83; 1×10^{-4} M $NaClO_4$, pK 8.28; 2.5×10^{-4} M $NaClO_4$, pK 8.39; 1×10^{-3} M $NaClO_4$, pK 8.70. It is clear that the binding of perchlorate has a pronounced effect on this equilibrium and hence that hemerythrin binds ClO_4^- strongly at a site near the iron. The effect of nitrate binding on this equilibrium is approximately half that of perchlorate. Similar results have been observed for other aquo-ligand equilibria.

The effect of perchlorate ion binding on the rate of formation of a ligand species is shown in Figure 3. The presence of a 20-fold excess of sodium perchlorate over protein iron concentration causes a large decrease in the rate of formation of the met-azidehemerythrin from met-aquohemerythrin. This phenomenon too clearly demonstrates that perchlorate ion is bound near the iron in this protein.

The techniques described do not permit us to see whether ligand anions that can be coordinated to the iron are also bound to the specific anion binding site. However electrophore-

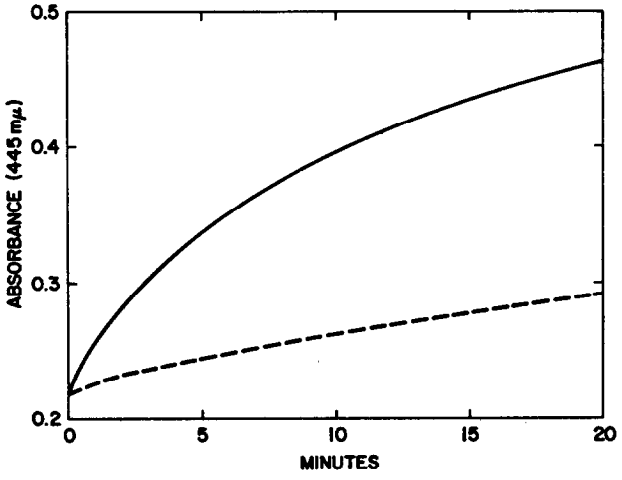

Figure 3. Rate of formation of met-azidehemerythrin from met-aquohemerythrin.

————0.00 M $NaClO_4$

---------8.35×10^{-3} M $NaClO_4$

Both solutions contained 3.46×10^{-4} M Fe, 5.25×10^{-4} M NaN_3; pH 7.0, 5°C.

tic work (Keresztes-Nagy and Klotz, I.M., unpublished results) indicates that chloride ion is bound to oxyhemerythrin in its native state.

Behavior at Sulfhydryl Site

Not only do ClO_4^- and NO_3^- affect the iron locus, but these ions also simultaneously "mask" the -SH group in its reaction with mercurials.

This was demonstrated by the following experiment. A stoichiometric amount of salyrganic acid, in relation to cysteine residues, was added to a solution of (7.0×10^{-4} M Fe) met-aquohemerythrin at pH 7.0. Immediately after the mercurial was dissolved, the solution was divided into two portions. To

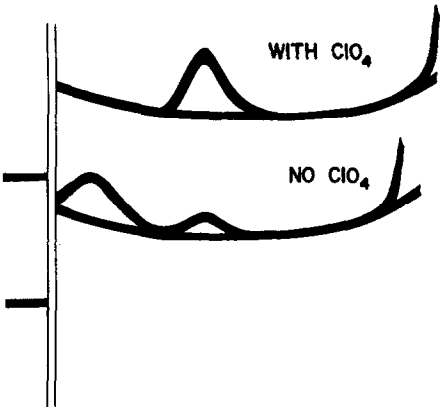

Figure 4. Sedimentation of met-aquohemerythrin. Both solutions contained 7.0×10^{-4} M Fe, 3.5×10^{-4} M salyrganic acid at pH 7.0, 5°C. Both diagrams from photographs taken 90 minutes after reaching speed of 59,780 RPM.

Upper - 9.1×10^{-4} M $NaClO_4$
Lower - 0.0 M $NaClO_4$

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one portion sodium perchlorate was added to a final concentration of 9.1×10^{-4} M. To the other portion the same amount of tris-cacodylate buffer was added to keep the ionic strengths the same for both solutions. After standing overnight at 4°C, the samples were simultaneously sedimented in the Spinco Model E Analytical Ultracentrifuge. As is evident in Figure 4 the sample without perchlorate was about 80% dissociated (into 2S particles) whereas that with perchlorate showed only non-dissociated octameric protein (6S particles). In other experiments essentially complete protection of the sulfhydryl was found when the concentration of perchlorate was the same as that of the cysteine in the protein. This indicates that a single perchlorate binding site (per subunit) provides the locus for binding ClO_4^- strongly and producing the effects observed.

Other sedimentation experiments, were carried out with nitrate as the -SH protective agent. At pH 7.0 nitrate protects against dissociation but not as effectively as perchlorate. This lessened effectiveness of NO_3^- with regard to -SH reactivity parallels its relative effectiveness at the iron site.

Titrations of the sulfhydryl group with p-chloromercuribenzoate by the method of Boyer (1954) have also shown markedly reduced rates of reaction in the presence of ClO_4^-. Much more extensive -SH titration studies are currently in progress elsewhere (S. Keresztes-Nagy, private communication).

The attenuating effects of these non-ligand anions on -SH reactivity stand in striking contrast to the marked enhancements in rates produced by iron-coordinating ligands such as azide (Keresztes-Nagy and Klotz, 1965).

Discussion

These observations indicate that hemerythrin binds ClO_4^- and a few other ions very strongly without their being coordinated to the Fe atoms. The non-ligand anion affects the kinetic and equilibrium behavior at the Fe locus, probably by electrostatic repulsive interactions. The non-ligand anion also affects the reactivity of the SH group. However, this interaction may not be wholly electrostatic for it has also been observed in titrations with uncharged N-ethylmaleimide, as well as with anionic mercurials. The stoichiometry of the effects of ClO_4^- indicates strongly that the effects described are produced by a single ClO_4^- bound at a single site (on each subunit). Since this ClO_4^- ion affects both the Fe locus and the SH locus we have herewith strong evidence that the oxygen-binding site and the single cysteine residue in hemerythrin are in close proximity.

References

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