KINETICS OF LIGAND BINDING, AMINO ACID SEQUENCE AND CRYSTALLIZATION OF CHLOROBIUM AND CHROMATIUM FLAVOCYTOCHROME C

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Introduction

Flavocytochromes c have been found in several species of phototrophic purple and green sulfur bacteria where they appear to function as sulfide dehydrogenases (1). Nucleophilic ligands such as sulfite, thiosulfate, cyanide, and mercaptans form adducts with the flavin of these proteins and the adducts in turn form charge transfer complexes with the protein (2). We previously measured the kinetics of adduct formation in Chromatium vinosum flavocytochrome c and showed that the reaction was complex (3). With sulfite, there is a rapid second-order bleach of flavin absorbance (with the associated appearance of a charge transfer band at 660 nm) followed at high pH by a slower recoloring reaction. Reaction with both sulfite and thiosulfate is apparently facilitated by protonation of a postulated histidine residue.

We have extended detailed characterization of the reaction of sulfite with flavocytochrome c to examples from Chlorobium thiosulfatophilum and Chromatium gracile to determine the extent of similarity and difference to Chromatium vinosum.

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Results and Discussion

For all three species of phototrophic bacterial flavocytochrome c, we observed a rapid bleach of flavin absorbance (adduct formation) by sulfite, which at low pH results in a relatively stable species, but at high pH, the protein recolors in a first-order process. The rapid bleach was second order at low sulfite concentrations, but became first order at high. C. gracile is intermediate between Chlorobium and C. vinosum in terms of the magnitude of both second- and first-order reactions with sulfite. Both reactions were pH dependent. This new finding of a change in the rate limiting step in the rapid bleach is a consequence of the higher sulfite concentrations used in the present study. Typically K_d ranges from 6-24 μM, complex formation from 9×10^3 to 6×10^4 $M^{-1}s^{-1}$ and the first-order isomerization from 100 to 200 s^{-1} for the three flavocytochromes c studied.

The recoloring reaction which was observed with sulfite at high pH in C. vinosum flavocytochrome also occurred with the Chlorobium and C. gracile proteins. The same pH effect was observed, but the C. gracile reaction was 3 times more rapid than C. vinosum and Chlorobium was a factor of 2 slower. This first-order reaction reflects a conformational change which places the flavin in an environment, which results in decomposition of the adduct perhaps through loss of the charge transfer interaction. This is a consequence of the reaction of sulfite at another site in the protein, such as at a disulfide. The pK of 8.4 previously observed in the C. vinosum reaction suggested the involvement of a cysteine residue (3). As a consequence of examining even lower sulfite concentrations than in the previous study, we find that with C. vinosum at pH 9, the recoloring reaction begins to show concentration dependence. This suggests that the postulated reaction with a disulfide is becoming rate limiting.

The recoloring reaction is over in less than two minutes at pH 8 and above, but at the highest sulfite concentrations this reaction is followed by a very slow rebleach of flavin absorbance complete in about 30 min or longer. This reaction occurs with all three species of protein; it is pH dependent, and also concentration dependent. This reaction is not likely to have physiological relevance, but it illustrates yet another conformation which the flavin can assume.

Available data suggests that the flavocytochromes can exist in several forms. These are summarized in equation 1 where for the present FC_1 and FC_2 as well as $adduct_1$ and $adduct_2$ represent species with different reactivities, and complex represents a collision complex prior to an isomerization leading to adduct formation.

$$\begin{matrix} & SO_3^= & & SO_3^= & SO_3^= & \\ FC_1 & \rightleftarrows & \text{complex} \rightleftarrows \text{adduct}_1 & \rightleftarrows & FC_2 - & \rightleftarrows \quad \text{adduct}_2 \end{matrix} \qquad (1)$$

A correlation between redox potentials of model flavins and reactivity with sulfite (4), and with flavoproteins (5) has been shown, which suggests that the flavin of flavocytochrome c has a different redox potential in each of its proposed conformations. Thus it can be argued that the flavin moiety of FC_1 and FC_2 have different redox potentials due to the presence of a sulfite modified disulfide near the flavin. This would explain the different kinetics of reaction with sulfite of FC_1 and FC_2.

We determined the amino acid sequence of the Chlorobium flavocytochrome heme subunit. It consists of 89 residues and is thus about the same size as that of Pseudomonas putida flavocytochrome c (78 residues), (6). From the location of the heme binding cysteine, histidine, and methionine residues, it is apparent that these proteins are homologous, although there is very little similarity otherwise (about 21%). In fact, the similarity is so low as to suggest convergence. In

spite of the fact that flavocytochromes are quite rare in nature, our results suggest that the chance association of cytochrome and flavoprotein to form a stable dimer may have occurred more than once over the course of evolution.

In a further attempt to compare these proteins and to determine the basis for the differences in their properties, we crystallized all three phototrophic flavocytochromes. C. vinosum flavocytochrome crystallization was reported by Salemme (7). Although that report was optimistic, the crystals were not suitable for high resolution X-ray analysis. Large, single crystals of the flavocytochrome isolated from Chromatium gracile were grown at room temperature. While the crystals were physically robust rods, they diffract only to very low resolution. Chlorobium thiosulfatophilum flavocytochrome was crystallized with large rod-shaped crystals appearing overnight. Again these crystals show diffraction maxima to only very low resolution. The poor quality of the flavocytochrome crystals, in terms of their diffraction patterns, suggests possible disorder in the crystalline lattices. Such disorder can often arise from microheterogeneity in the protein sample itself. Conformational heterogeneity is another possible source of disorder particularly if the protein under investigation is quite flexible. With this in mind, we are currently exploring approaches which might force the protein to adopt a single conformation.

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