Interaction of *Chromatium vinosum* flavocytochrome *c*-552 with cytochromes *c* studied by affinity chromatography

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Flavocytochrome *c*-552, isolated from the photosynthetic purple sulfur bacterium *Chromatium vinosum*, after linkage to an Affigel 10-affinity matrix, will bind equine and yeast mitochondrial cytochromes *c*, *C. vinosum* cytochrome *c*-550 and cytochrome c_2 from the photosynthetic purple non-sulfur bacterium *Rhodopseudomonas viridis*. Similarly, an equine cytochrome *c*-Sepharose 4B affinity column will bind the *C. vinosum* flavocytochrome *c*-552 and its separated heme-containing subunit. These results and the correlation between the effect of ionic strength on binding of the flavocytochrome *c*-552 to cytochrome *c* and on the sulfide:cytochrome *c* oxidoreductase activity catalyzed by *C. vinosum* flavocytochrome *c*-552 support the idea of the involvement of an electrostatic complex between the two proteins during sulfide oxidation.

Flavocytochrome c-552 Chromatium vinosum *Cytochrome c* *Affinity chromatography*

1. INTRODUCTION

Considerable evidence exists that a soluble flavocytochrome *c*-552 participates in the oxidation of sulfide to sulfur in the photosynthetic purple sulfur bacterium *Chromatium vinosum* [1–3]. The *C. vinosum* flavocytochrome *c*-552 (67 kDa) is a dimeric protein containing non-equivalent subunits: A 46-kDa peptide containing a single FAD and a 21-kDa peptide containing two hemes *c* [2,4]. Fukumori and Yamanaka [2] demonstrated that the *C. vinosum* flavocytochrome *c*-552 exhibited sulfide:cytochrome *c* oxidoreductase activity with equine mitochondrial cytochrome *c* and several high-potential, bacterial cytochromes *c* serving as electron acceptors. At that time, no soluble, high-potential *c*-type cytochromes had been detected in *C. vinosum*. More recently, *C. vinosum* has been shown to contain cytochrome *c*-550 [5–8], a soluble, high-potential cytochrome, related to mitochondrial cytochrome *c* [6]. This finding suggests that model studies using equine cytochrome *c* as an electron acceptor in place of the endogenous *C. vinosum* cytochrome *c*-550 can provide useful information concerning the mechanism of the *C. vinosum* flavocytochrome *c*-552 catalyzed oxidation of sulfide.

Evidence obtained in our laboratory [3], supported by the recent observations of Meyer et al. [9], suggested that the sulfide:cytochrome *c* oxidoreductase reaction catalyzed by the *C. vinosum* flavocytochrome *c*-552 involves an electrostatic complex between the two proteins. Below we present evidence, based on affinity chromatography techniques, supporting the hypothesis that the *C. vinosum* flavocytochrome *c*-552 does form a complex with a number of high-potential cytochromes *c*, including *C. vinosum* cytochrome *c*-550, that can serve as electron acceptors for sulfide oxidation. In addition, we have obtained evidence that the major site of interaction between cytochrome *c* and flavocytochrome *c*-552 involves the 21-kDa heme subunit of the *C. vinosum* flavocytochrome *c*-552.

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2. MATERIALS AND METHODS

C. vinosum flavocytochrome *c*-552 was purified by a slight modification of the method of Bartsch and Kamen [4], using Sephacryl S-200 for the gel filtration steps and omitting ammonium sulfate precipitation. The resulting protein (in its oxidized form) had an absorbance ratio (A_{280}:A_{410}) ranging from 0.50 to 0.56 (for different preparations) and showed only two Coomassie brilliant blue-staining peptide bands (46 and 21 kDa, corresponding to the flavin and heme-containing subunits, respectively [2]) after polyacrylamide gel electrophoresis (PAGE) in the presence of SDS. Gel electrophoresis was performed on 12% slab gels (1.5 mm thickness) prepared according to O'Farrell [10]. The heme and flavin subunits of the *C. vinosum* flavocytochrome *c*-552 were purified according to Vorkink [11] and Brown [12], respectively. The heme subunit preparations had A_{280}:A_{410} ratios < 0.20, lacked any evidence of flavin absorbance features, and exhibited a single Coomassie blue-staining band (21 kDa) after SDS-PAGE. The flavin subunit preparations had spectra essentially identical to that described by Brown [12]. The absence of any feature at 412 nm attributable to the Soret band of the oxidized cytochrome [2,4,11,12] and the fact that the preparation exhibited only a single Coomassie blue-staining band (46 kDa) after SDS-PAGE demonstrated the freedom of these preparations from any residual heme subunit.

Cytochromes c_2 from *Rhodospirillum rubrum* and *Rhodopseudomonas viridis* were purified according to Bartsch [13]. Both cytochromes exhibited single peptide bands following SDS-PAGE. *C. vinosum* HiPIP and cytochrome *c'* were also purified as in [13]. *C. vinosum* cytochrome *c*-550 was partially purified as described in [8]. Equine cytochrome *c* (type VI) and *Saccharomyces cerevisiae* cytochrome *c* were obtained from Sigma. The former was purified further by ion-exchange chromatography on CM-Sephadex while the latter was used without further purification. Complete trifluoroacetylation of equine cytochrome *c* was accomplished using the method of Fanger and Harbury [14].

Affigel-10 was obtained from Bio-Rad and cyanogen bromide-activated Sepharose 4B from Pharmacia. Coupling of the various proteins utilized in these studies to the two affinity matrices was accomplished following procedures outlined by the manufacturers [15,16]. The equine cytochrome *c*-Sepharose 4B matrix was prepared using 52 mg cytochrome and 1.75 g cyanogen bromide-activated Sepharose 4B. The *C. vinosum* flavocytochrome *c*-552–Affigel 10 matrix was prepared using 330 nmol cytochrome and 3 ml Affigel 10.

Absorbance spectra, at a spectral resolution of 1.0 nm, were obtained using Aminco DW-2a and Perkin-Elmer Lambda 5 spectrophotometers. Sulfide:cytochrome *c* oxidoreductase activity was measured using the Aminco DW-2a spectrophotometer, as described [3].

3. RESULTS

It seemed likely that if the *C. vinosum* flavocytochrome *c*-552 forms a complex with its cytochrome *c* reaction partner during the flavocytochrome *c*-552-catalyzed oxidation of sulfide, then *c*-type cytochromes that can function as electron acceptors in this reaction would bind to a flavocytochrome *c*-552–Affigel 10 affinity column. Fig.1 shows that this is indeed the case for

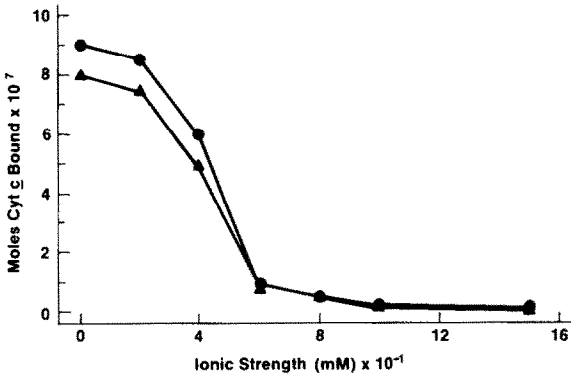

Fig.1. Binding of equine and yeast cytochromes *c* to a *C. vinosum* flavocytochrome *c*-552–Affigel 10 affinity column. Either 900 nmol equine cytochrome *c* (●) or 800 nmol yeast (*S. cerevisiae*) cytochrome *c* (▲) in 20 mM Tris buffer (pH 8.0) was applied to the affinity column (bed volume 3.0 ml) which had been pre-equilibrated with the same buffer. The ionic strength of the eluting buffer was increased by NaCl addition. 2.0-ml fractions were collected. Total recovery of each cytochrome was approx. 95%.

equine and *S. cerevisiae* cytochromes *c*. Binding of both partially purified *C. vinosum* cytochrome *c*-550, the putative native electron acceptor in the flavocytochrome *c*-552-catalyzed oxidation of sulfide, and cytochrome c_2 isolated from the photosynthetic bacterium *Rps. viridis* (which, like cytochrome *c*-550, can serve as an effective electron acceptor) to the flavocytochrome *c*-552–Affigel 10 affinity column was also observed (not shown). Surprisingly, cytochrome c_2 isolated from the photosynthetic bacterium *R. rubrum*, which can also serve as a good electron acceptor for sulfide oxidation [2], did not bind to the flavocytochrome *c*-552–Affigel 10 affinity column. In control experiments, an Affigel-10 column containing no flavocytochrome *c*-552 but instead had the *N*-hydroxysuccinimide covalent linking groups blocked with glycine, showed no binding of equine or *S. cerevisiae* cytochromes *c*, *C. vinosum* cytochrome *c*-550 or *Rps. viridis* cytochrome c_2. These controls establish that the 4 cytochromes *c* bind to the affinity ligand, flavocytochrome *c*-552, rather than non-specifically to the Affigel-10 agarose matrix. In an attempt to document further the specificity of the interactions of the flavocytochrome *c*-552–Affigel 10 affinity column, it was demonstrated that neither cytochrome *c'* nor HiPIP, two soluble electron transfer proteins isolated from *C. vinosum*, bound to the column.

Earlier observations in our laboratory [3] suggested that the interaction between *C. vinosum* flavocytochrome *c*-552 and cytochrome *c* was dominated by electrostatic forces. The results in fig.1, showing that *c*-type cytochromes that bind to the flavocytochrome *c*-552–Affigel 10 affinity column can be eluted from the column as the ionic strength of the eluting buffer is increased, is consistent with this hypothesis. Fig.2 shows that the sulfide : cytochrome *c* oxidoreductase activity of *C. vinosum* flavocytochrome *c*-552 diminishes over approximately the same range of ionic strengths required to disrupt the binding of cytochromes *c* to the flavocytochrome *c*-552–Affigel 10 affinity column. Previous work in our laboratory had implicated the positive charges on cytochrome *c* lysine residues in the interaction of the cytochrome with *C. vinosum* flavocytochrome *c*-552 [3]. Completely trifluoroacetylated cytochrome *c*, in which all lysine ϵ-amino groups have been acetylated [14], not only cannot serve as an electron acceptor for sulfide oxidation [3] but also fails to bind to the flavocytochrome *c*-552–Affigel 10 affinity column (not shown).

Additional evidence for complex formation between the *C. vinosum* flavocytochrome *c*-552 and cytochrome *c* came from the observation, shown in fig.3, that the flavocytochrome *c*-552 binds to an equine cytochrome *c*-Sepharose 4B affinity column. As was the case in the experiments of fig.1, flavocytochrome *c*-552 could be eluted from the

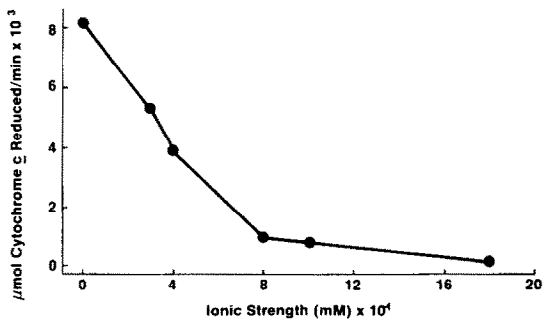

Fig.2. Effect of ionic strength on the sulfide:cytochrome *c* oxidoreductase activity of *C. vinosum* flavocytochrome *c*-552. The reaction mixtures contained 20 μM equine cytochrome *c*, 20 μM Na_2S and 40 nM flavocytochrome *c*-552 in 10 mM Tris buffer (pH 8.0). Aliquots of 2 M NaCl were used to adjust the ionic strength.

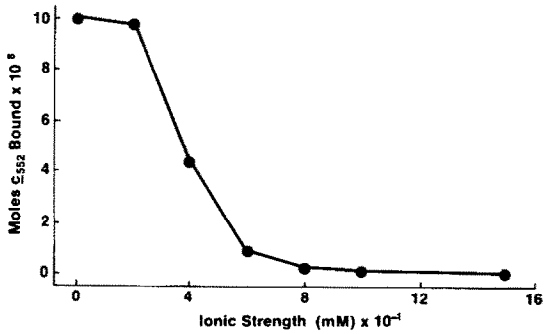

Fig.3. Binding of *C. vinosum* flavocytochrome *c*-552 to an equine cytochrome *c*-Sepharose 4B affinity column. 200 nmol flavocytochrome *c*-552 in 20 mM Tris buffer (pH 8.0) was applied to the affinity column (bed volume 5.3 ml) which had been pre-equilibrated with the same buffer. Other conditions as in fig.1. Total recovery of the cytochrome was 98%.

cytochrome *c*-Sepharose 4B affinity column by increasing the ionic strength of the eluting buffer. A Sepharose-4B column, containing no cytochrome *c*, in which all the cyanogen bromide covalent linking groups had been blocked with glycine, showed no binding of the *C. vinosum* flavocytochrome *c*-552. This result suggests the binding of flavocytochrome *c*-552 to the cytochrome *c* affinity column results from interactions with the equine cytochrome *c* ligand rather than the Sepharose matrix.

The availability of the cytochrome *c*-Sepharose 4B affinity column as a probe for complex formation with the *C. vinosum* flavocytochrome *c*-552 allowed us to investigate which of the flavocytochrome *c*-552 subunits is involved in complex formation between the two proteins. Fig.4 shows that the heme-containing subunit of the *C. vinosum* flavocytochrome *c*-552 binds to the cytochrome *c*-Sepharose 4B affinity column at low ionic strength and is eluted from the column when the ionic strength is raised by addition of 150 mM NaCl to the eluting buffer. No binding of the flavin-containing subunit of the flavocytochrome *c*-552 to the cytochrome *c*-Sepharose 4B affinity column was observed.

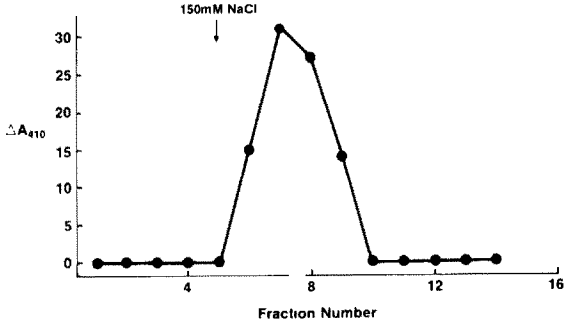

Fig.4. Binding of the heme-containing subunit of *C. vinosum* flavocytochrome *c*-552 to an equine cytochrome *c*-Sepharose 4B affinity column. 68 nmol heme subunit was loaded on the affinity column as described in fig.3. The column was then washed with 20 mM Tris buffer (pH 8.0) and, where indicated, the elution buffer was changed to 20 mM Tris (pH 8.0) plus 150 mM NaCl. Total recovery of the heme subunit was 98%.

4. DISCUSSION

The data presented above provide considerable evidence for the hypothesis [3] that *C. vinosum* flavocytochrome *c*-552 forms a complex with a number of *c*-type cytochromes that can serve as electron acceptors in the sulfide:cytochrome *c* oxidoreductase activity exhibited by the *C. vinosum* enzyme. Of particular interest was the observation that the endogenous, soluble, high-potential *C. vinosum* cytochrome *c*-550 could both bind to a flavocytochrome *c*-552–Affigel 10 affinity column and serve as an electron acceptor for sulfide oxidation. This is the first demonstration that the native *C. vinosum* cytochrome can interact with flavocytochrome *c*-552 and can actually participate as an electron acceptor in this in vitro model for a redox reaction of considerable importance in the metabolism of *C. vinosum* [1]. Unfortunately, the difficulty of obtaining adequate amounts of cytochrome *c*-550 and its instability [7,8] prevented us from characterizing the interaction of the two *C. vinosum* proteins in greater detail. It is not yet clear why *R. rubrum* cytochrome c_2, which can serve as an electron acceptor in the flavocytochrome *c*-552-catalyzed oxidation of sulfide [2], does not bind to the flavocytochrome *c*-552–Affigel 10 affinity column. This is in contrast to equine and *S. cerevisiae* cytochromes *c*, *C. vinosum* cytochrome *c*-550 and *Rps. viridis* cytochrome c_2, all of which both serve as electron acceptors during sulfide oxidation and bind to the flavocytochrome *c*-552–Affigel 10 affinity column. It may simply be that complex formation with the *R. rubrum* cytochrome c_2 is weaker or more sensitive to ionic strength than in the case of the other cytochrome complexes.

The observation that the sulfide:cytochrome *c* oxidoreductase activity of the *C. vinosum* flavocytochrome *c*-552-catalyzed reaction falls off with increasing ionic strength (see fig.2) over approximately the same range as required to disrupt the cytochrome *c*:flavocytochrome *c*-552 complex (see figs 1 and 3) is consistent with the idea that complex formation is required for activity and that electrostatic forces play a large role in stabilizing the complex. (We have been unable to confirm our earlier observation [3] of a slight increase in activity with increasing ionic strength at very low ionic strengths. Ionic strength profiles essentially iden-

tical to those of fig.2 have been recently obtained in other laboratories (F. Millett and H.R. Bosshard, personal communications).) The observation that acetylation of the lysine groups of cytochrome *c* eliminates both binding to a flavocytochrome *c*-552–Affigel 10 affinity column (see above) and the ability to serve as an electron acceptor during sulfide oxidation [3] suggests that positive charges on cytochrome *c* lysine residues make a major contribution to complex formation. The putative complementary negative charges on the flavocytochrome *c*-552 would appear to be located predominantly on its heme subunit, as binding of the heme subunit but not the flavin subunit of the flavocytochrome *c*-552 to equine cytochrome *c* can be observed by the affinity chromatography technique.

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REFERENCES

[1] Trüper, H.G. and Fischer, U. (1982) Phil. Trans. Roy. Soc. Lond. B298, 529–542.
[2] Fukumori, Y. and Yamanaka, T. (1979) Biochem. J. 85, 1405–1414.
[3] Gray, G.O. and Knaff, D.B. (1982) Biochim. Biophys. Acta 680, 290–296.
[4] Bartsch, R.G. and Kamen, M.D. (1960) J. Biol. Chem. 235, 825–831.
[5] Van Grondelle, R., Duysens, L.N.M., Van der Wal, J.A. and Van der Wal, H.N. (1977) Biochim. Biophys. Acta 461, 188–201.
[6] Knaff, D.B., Whetstone, R. and Carr, J.W. (1980) Biochim. Biophys. Acta 590, 50–58.
[7] Tomiyama, Y., Doi, M., Takamiya, K. and Nishimura, M. (1983) Plant Cell Physiol. 24, 11–16.
[8] Gray, G.O., Gaul, D.F. and Knaff, D.B. (1983) Arch. Biochem. Biophys. 222, 78–86.
[9] Meyer, T.E., Vorkink, W.P., Tollin, G. and Cusanovich, M.A. (1985) Arch. Biochem. Biophys. 236, 52–58.
[10] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[11] Vorkink, W.P. (1972) PhD Dissertation, University of Arizona.
[12] Brown, S.L. (1981) MS Thesis, University of Arizona.
[13] Bartsch, R.G. (1971) Methods Enzymol. 23, 344–363.
[14] Fanger, M.W. and Harbury, H.A. (1965) Biochemistry 4, 2541–2545.
[15] Bio-Rad Laboratories, Technical Bulletin 1085.
[16] Affinity Chromatography, Principles and Methods (1979) Pharmacia Fine Chemicals.