Flavocytochrome \(c_{552}\) from *Chromatium vinosum* catalyzes the oxidation of sulfide to sulfur using a soluble c-type cytochrome as an electron acceptor. Mitochondrial cytochrome \(c\) forms a stable complex with flavocytochrome \(c_{552}\) and may function as an alternative electron acceptor \emph{in vitro}. The recognition site for flavocytochrome \(c_{552}\) on equine cytochrome \(c\) has been deduced by differential chemical modification of cytochrome \(c\) in the presence and absence of flavocytochrome \(c_{552}\) and by kinetic analysis of the sulfide:cytochrome \(c\) oxidoreductase activity of \(m\)-trifluoromethylphenylcarbamoyl-lysine derivatives of cytochrome \(c\). As with mitochondrial redox partners, interaction occurs around the exposed heme edge at the “front face” of cytochrome \(c\). However, the domain recognized by flavocytochrome \(c_{552}\) seems to extend to the right of the heme edge, whereas the site of interaction with mitochondrial cytochrome \(c\) oxidase and reductase is more to the left.

*K* but not \(V_{max}\) of the electron transfer reaction with mitochondrial cytochrome \(c\) increases with increasing ionic strength. The correlation of chemical modification and ionic strength dependence data indicates that the electrostatic interaction between the two hemoproteins involves fewer ionic bonds than that with other redox partners of cytochrome \(c\).

The mechanism of electron transfer by eukaryotic mitochondrial cytochrome \(c\) has been shown to involve the formation of complexes with its reaction partners that are stabilized by electrostatic interactions (1, 2). Extensive chemical modification studies have shown that six to eight highly conserved lysine residues surrounding the heme crevice of cytochrome \(c\) are involved in the interaction with cytochrome \(c\) oxidase (2–6), the cytochrome \(b_6\) complex (7–10), cytochrome \(c\) peroxidase (11, 12), cytochrome \(b_6\) (13), sulfite oxidase (14, 15), and adrenodoxin (16). The cytochrome \(c\)-binding sites on cytochrome \(b_6\) and cytochrome \(c\) peroxidase have been deduced from model building studies based on the crystallographic structures (17, 18) and from chemical modification and cross-linking studies (19–22). There are negatively charged carboxylates on these proteins that form complementary charge-pair interactions with the lysines surrounding the heme crevice of cytochrome \(c\) (17, 18, 20, 21).

*Chromatium vinosum*, like all photosynthetic purple sulfur bacteria, possesses the ability to oxidize sulfide to sulfur (23). Fukumori and Yamanaka (24) demonstrated that flavocytochrome \(c_{552}\) (\(M_r = 67,000\)) isolated from *C. vinosum* (25) has sulfide:cytochrome \(c\) oxidoreductase activity and proposed that this flavocytochrome was the enzyme responsible for catalyzing the oxidation of sulfide to sulfur observed \emph{in vivo}. This hypothesis was made more plausible by the demonstration that sulfur was in fact the major product resulting from the \emph{in vitro} oxidation of sulfide catalyzed by flavocytochrome \(c_{552}\) with equine mitochondrial cytochrome \(c\) serving as the electron acceptor (26) however, see Ref. 41). One remaining difficulty with the hypothesis of Fukumori and Yamanaka was the use of equine cytochrome \(c\) as a model electron acceptor in experiments designed to support their proposal (24). At that time *C. vinosum*, unlike representative species of photosynthetic purple nonsulfur bacteria (1), was thought not to contain a soluble c-type cytochrome related to mitochondrial cytochrome \(c\). However, recent evidence (27–31) indicating that *C. vinosum* does in fact contain a soluble high-potential cytochrome \(c\) similar to mitochondrial cytochrome \(c\) has removed this last objection to assigning the role of a sulfide:cytochrome \(c\) oxidoreductase to the *C. vinosum* flavocytochrome \(c_{552}\).

As the sulfide:cytochrome \(c\) oxidoreductase reaction catalyzed by flavocytochrome \(c_{552}\) also appears to involve an electrostatic complex between cytochrome \(c\) and the *C. vinosum* flavocytochrome \(c_{552}\) (26, 31, 32), it is of interest to know whether the same lysine residues on equine cytochrome \(c\) that demarcate the site of interaction with its mitochondrial reaction partners are involved in interacting with the nonphysiological reaction partner flavocytochrome \(c_{552}\). Two independent techniques were chosen to explore this possibility, differential chemical modification (33) of cytochrome \(c\) lysine residues in the presence and absence of flavocytochrome \(c_{552}\) and examination of the effect of chemical modification of specific cytochrome \(c\) lysine residues (7) on the kinetic parameters of the sulfide:cytochrome \(c\) oxidoreductase reaction catalyzed by the *C. vinosum* enzyme.

**MATERIALS AND METHODS**

*C. vinosum* flavocytochrome \(c_{552}\) was purified by a slight modification of the method of Bartch (34), using Sephacryl S-200 for the gel filtration steps and omitting ammonium sulfate precipitation. The resulting protein (in its oxidized form) has an absorbance ratio \((\lambda_{max}/\lambda_{min})\) ranging from 0.50 to 0.60 (for different preparations) and showed only two Coomassie Brilliant Blue-staining polypeptides (\(M_r = 46,000\) and 21,000, corresponding to the flavin and heme-containing subunits, respectively (24)) after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate on 10–22% gradient slab
Flavocytochrome c552-Cytochrome c Complex

gels (1.5 mm thickness) prepared according to O'Farrell (38). Equine cytochrome c (Type VI, Sigma) was further purified prior to use by chromatography on CM-Sephadex (36). The m-trifluoromethylphenylcarbamoyl-lysine-modified cytochrome c derivatives were prepared and purified as described previously (3).

Differential chemical modification of cytochrome c in the presence and absence of flavocytochrome c552 was performed essentially as before (6, 10, 33). Ferricytochrome c (74 μM) and flavocytochrome c552 (78 μM) in 10 mM triethanolamine-HCl buffer, pH 8.2, were reacted at 0 °C with 2μH-acetic anhydride (0.3 mM, 7.5 C/minol). In a parallel experiment cytochrome c was acetylated in the absence of flavocytochrome c552. All other conditions being the same. [3H]Phenylalanine (200 μM) was included in both experiments to serve as an internal standard nucleophile (6, 33). [3H]Acetylated cytochrome c was separated from flavocytochrome c552 by chromatography on a column (0.9 x 4 cm) of CM-cellulose (Whatman CM32) that had been equilibrated with 50 mM phosphate buffer, pH 7.0. Cytochrome c was eluted from this column by 0.2 M phosphate buffer, pH 7.0. Tritium-labeled cytochrome c from the two parallel experiments had less than 0.3 mol of [3H]acetyl group/mol of protein, on average. A low degree of H labeling guaranteed that R values (see below) truly reflected the influence of complex formation on chemical reactivity (32).

Equimolar amounts of the two 3H-labeled cytochrome c derivatives (450 nmol) were each mixed with equimolar amounts of uniformly 14C-acetylated cytochrome c derivatives (53 nmol, 89 Ci/mol). The mixtures were denatured and treated with nonradioactive acetic anhydride in excess in order to get uniformly N'-acylated derivatives (6, 10). The derivatives were digested with chymotrypsin and thermolysin, respectively. Chymotryptic and thermolytic peptides were separated by high performance liquid chromatography on an Aqua- pore RP-300 reversed-phase column (Brownlee, Santa Barbara, CA), using acetonitrile gradients in 0.1% trifluoroacetic acid (equipment and further details as in Refs. 20 and 37).

Purified peptides were analyzed for amino acid composition, N-terminal residue, and 14C/14C radioactivity ratio. Peptides with several N'-acytlylsine residues were degraded by a modified Edman method (38) to get 14C/14C ratios of single N'-acytlylsines. The 14C/14C ratios were corrected by the 14C/14C ratio of the acetylated standard nucleophile (6, 33). The corrected 14C/14C ratio of a N'-acytlylsine labeled in free cytochrome c was divided by the correleted 14C/14C ratio of the same residue labeled in the cytochrome c:flavocytochrome c552 complex. The resulting number was called reactivity ratio R (33). Based on our past experience, R values are in error by 10–15% (6, 20, 39).

Absorbance spectra were measured on Aminco DW-2a and Perkin-Elmer Lambda Five spectrophotometers at a spectral resolution of 1.0 nm. Sulfide:cytochrome c oxidoreductase activity was monitored using acetonitrile gradients in 0.1% trifluoroacetic acid (equipment and further details as in Refs. 20 and 37).

RESULTS

We have previously demonstrated (26) that, as expected if an electrostatic protein:protein complex involved, the sulfide:cytochrome c oxidoreductase activity of C. vinosum flavocytochrome c552 falls off with increasing ionic strength. As can be seen from Fig. 1, increasing the ionic strength from 2.5 to 100 mM had no effect, within the experimental uncertainty, on the maximum velocity but resulted in a greater than 4-fold increase in the Km for cytochrome c. The absence of any effect of ionic strength on turnover number suggests that it is probably the binding of cytochrome c to flavocytochrome c552 rather than the electron transfer per se that is sensitive to ionic strength. Furthermore, the observed increase in Km with increasing ionic strength is consistent with the hypothesis that the forces involved in the association of the two proteins are partially electrostatic in nature.

To identify the specific residues involved in the electrostatic interaction, the chemical reactivity of lysine residues of cytochrome c toward acetic anhydride was compared in free and flavocytochrome c552-bound cytochrome c by differential chemical modification. The reactivity ratios R for 16 of the 19 lysine residues of cytochrome c have been determined (Table I and Fig. 2, filled bars). As discussed elsewhere (33), R is a lower estimate of the true degree by which the chemical reactivity is affected through formation of the electron transfer complex. When cytochrome c bound to flavocytochrome c552, lysines 8, 13, 27, 79, 86, and 87 became significantly less reactive. All other residues remained about equally reactive or were, at most, slightly less reactive (lysines 5 and 88). The
significant less reactive lysines were all located on the front surface of the molecule, surrounding the exposed edge of the heme (Fig. 3).

Modification of each of five specific lysine residues on the front of cytochrome c (residues 8, 13, 27, 72, and 79) to form \( m \)-trifluoromethylphenylcarbamoyl derivatives resulted in each case in a decreased rate of cytochrome c reduction (Table II). In a control experiment, modification with the same modifying group of a lysine residue on the back of cytochrome c (residue 100) had no effect (within the experimental uncertainties) on the rate of cytochrome c reduction. Modification of front-side lysine residues had no effect (within the experimental uncertainties) on \( V_{\text{max}} \) but did result in significant increases in \( K_m \) (Table II). Modification of cytochrome c residues 72, 79, and 13 had the greatest effect on \( K_m \) for the S°:cytochrome c oxidoreductase activity of flavocytochrome c652. These results are not only consistent with the involvement of these front-side cytochrome c lysine residues in the association of the two proteins during the reduction of cytochrome c, but they are also in excellent agreement with the data from differential chemical modification (compare Tables I and II).

**DISCUSSION**

The experiments reported here demonstrate that the reaction between horse heart cytochrome c and C. vinosum flavocytochrome c652 depends on the formation of a complex stabilized by electrostatic interactions and involving the front side of cytochrome c. The latter conclusion follows from differential chemical modification of lysine residues in the presence and absence of flavocytochrome c652 and from the kinetic properties of \( m \)-trifluoromethylphenylcarbamoyl-lysine derivatives of cytochrome c. The two techniques are complementary and have been applied before to the elucidation of electron transfer sites (2-4, 6, 7, 9, 10, 12-14, 16). The differential chemical modification method is based on the assumption that the formation of an electron transfer complex will reduce the chemical reactivity of those lysines that are involved in ionic interaction with the reaction partner (see Refs. 10 and 33 for detailed discussion). The disadvantage of this method, however, is that the data do not relate to the electron transfer process proper. On the other hand, kinetic analysis of cytochrome c derivatives modified at a single lysine residue gives direct information on the involvement of that residue in the reaction. The major disadvantage of this technique is the possibility that the specific modification might cause a change in conformation at a remote site. Nevertheless, we have previously shown that these derivatives, which change the charge on a single amino acid residue from +1 to 0, have the same redox potential, visible absorption spectrum, optical dispersion spectrum, and proton NMR spectrum as native cytochrome c (3).

The combined results from differential chemical modification and kinetic analysis of lysine-substituted cytochrome c derivatives indicate that residues 8, 13, 27, 72, 79, 86, and 87 are taking part in complex formation with flavocytochrome c652 (Tables I and II, Fig. 2). The seven residues form a ring around the heme cleft, they are highly conserved, and they have been shown to take part to varying degrees in the interaction with several different reaction partners of cytochrome c. Hence, the approximate binding area for flavocytochrome c652 is equal to that for any other electron transfer partner tested so far (2-4, 6-16). However, there exist some small but possibly significant differences (Fig. 3, Table III).

First, flavocytochrome c652 interferes to a lesser degree with acetylation of front-side lysine residues. This is likely to be
due to a higher dissociation constant of the flavocytochrome 
C552cytochrome c complex as compared to the mitochondrial complexes. A more important difference, however, follows from the comparison of the relative heights of R values shown in Fig. 2. Flavocytochrome C552 inhibits the acetylation of residues 27 and 79, whereas the same two residues are only slightly protected by the mitochondrial reaction partners. Lysine 72, on the other hand, is much more shielded by the mitochondrial proteins than by flavocytochrome C552. This difference may possibly indicate that flavocytochrome C552 recognizes an area to the right of the heme cleft (Lys 72) and toward the lower front face (Lys 79; compare the relative heights of filled and open bars of Fig. 2). Incidentally, different extensions of electron transfer sites have been deduced for other redox partners of cytochrome c by kinetic analysis of lysine-substituted cytochrome c derivatives (14, 39). Such differences may reflect different structures of the complementary sites on cytochrome c's reaction partners and might even indicate slightly different intermolecular electron pathways.

Second, modification of front-side lysines affects the reaction with flavocytochrome C552 to a somewhat smaller extent than the reactions of cytochrome c with other molecules (Table III). The dependence of this reaction on intrinsic strength is also somewhat smaller, indicating that the electrostatic interaction between cytochrome c and flavocytochrome C552 is smaller than that involving the other partners of cytochrome c. A semi-empirical relationship has been developed for the electrostatic interactions of cytochrome c with its redox partners that is based on a correlation of chemical modification and intrinsic strength dependence data (40). This relationship predicts that there are the equivalent of about four complementary charge-pair interactions between lysine amino groups on cytochrome c and carboxylate groups on flavocytochrome C552. This compares to about six for the interactions of cytochrome c with cytochrome b and sulfite oxidase and about eight for the interactions with cytochrome b and cytochrome c oxidase.

In conclusion, the general pattern of complex formation and of intrinsic strength dependence of the electron transfer reaction follows that observed with the mitochondrial redox partners of cytochrome c. However, the recognition site for flavocytochrome C552 on cytochrome c is not identical to that for mitochondrial oxidase and reductase. The difference might reflect either the different structures of the electron transfer sites of the mitochondrial and C. vinosum proteins, or the involvement of different residues of cytochrome c in the actual electron transfer process, or both. Clarification of this important point has to await a closer analysis of the intermolecular interface of the different electron transfer complexes.

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