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The use of a water-soluble carbodiimide to study the interaction between *Chromatium vinosum* flavocytochrome *c*-552 and cytochrome *c*

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The interaction between horse heart cytochrome *c* and *Chromatium vinosum* flavocytochrome *c*-552 was studied using the water-soluble reagent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). Treatment of flavocytochrome *c*-552 with EDC was found to inhibit the sulfide:cytochrome *c* reductase activity of the enzyme. SDS gel electrophoresis studies revealed that EDC treatment led to modification of carboxyl groups in both the M_r 21000 heme peptide and the M_r 46000 flavin peptide, and also to the formation of a cross-linked heme peptide dimer with an M_r value of 42000. Both the inhibition of sulfide:cytochrome *c* reductase activity and the formation of the heme peptide dimer were decreased when the EDC modification was carried out in the presence of cytochrome *c*. In addition, two new cross-linked species with M_r values of 34000 and 59000 were formed. These were identified as cross-linked cytochrome *c*-heme peptide and cytochrome *c*-flavin peptide species, respectively. Neither of these species were formed in the presence of a cytochrome *c* derivative in which all of the lysine amino groups had been dimethylated, demonstrating that EDC had cross-linked lysine amino groups on native cytochrome *c* to carboxyl groups on the heme and flavin peptides. A complex between cytochrome *c* and flavocytochrome *c*-552 was required for cross-linking to occur, since ionic strengths above 100 mM inhibited cross-linking.

Introduction

The photosynthetic purple sulfur bacteria *Chromatium vinosum* is able to oxidize sulfide to elemental sulfur [1]. Fukumori and Yamanaka [2] demonstrated that a flavocytochrome *c*-552 (M_r = 67 kDa) isolated from *C. vinosum* [3] has sulfide:cytochrome *c* oxidoreductase activity and proposed that this flavocytochrome was the enzyme responsible for catalyzing the oxidation of sulfide to sulfur observed in vivo. This hypothesis is supported by the finding of Gray and Knaff [4] that sulfur was in fact the major product resulting from the in vitro oxidation of sulfide catalyzed by flavocytochrome *c*-552 with horse heart cytochrome *c* serving as the electron acceptor. Recent evidence [5–8] indicates that *C. vinosum* contains a soluble, high-potential cytochrome, cytochrome *c*-550, that could serve as electron acceptor in vivo. This endogenous cytochrome *c*-550 has properties similar to those of horse heart cytochrome *c*, and thus in vitro studies of the mechanism of flavocytochrome *c*-552 using horse heart cytochrome *c* as electron acceptor appear to be justified.

Flavocytochrome *c*-552 is an attractive enzyme for fundamental studies of electron transfer mechanisms because it is one of the few soluble proteins

Abbreviations: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Mops, 4-morpholinepropanesulfonic acid.

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that contains more than one type of redox prosthetic group. Fukumori and Yamanaka [2] have separated the enzyme into a flavoprotein subunit with an M_r of 46 000, and a smaller subunit with an M_r of 21 000 that contains two covalently bound heme *c* groups. Neither of the isolated subunits has any sulfide : cytochrome *c* reductase activity by itself. Cusanovich and Tollin [9] found that the rate for intramolecular electron transfer between the flavin and heme was at least $1.4 \cdot 10^6$ s^{-1}, and suggested a mechanism in which sulfide is oxidized to sulfur at the flavin subunit, and the electrons are then transferred to the heme subunit and thence to cytochrome *c*. Gray and Knaff have shown that flavocytochrome *c*-552 forms a complex with horse heart cytochrome *c* that is stabilized by electrostatic interactions [4]. We have recently discovered that specific modification of the lysine amino groups at residues 13, 27, 72 and 79 on cytochrome *c* significantly increased the Michaelis constant for the reaction with flavocytochrome *c*-552. These residues are part of the ring of highly conserved lysines surrounding the heme crevice of cytochrome *c* that have been shown to form the binding site for all the electron-transport partners of cytochrome *c*, including cytochrome oxidase [10–12], cytochrome c_1 [12–14], cytochrome *c* peroxidase [15,16] and cytochrome b_5 [17]. The cytochrome *c* binding sites on cytochrome b_5 and cytochrome *c* peroxidase have been deduced from model building studies based on the crystallographic structures [18,19]. There are negatively charged carboxylates surrounding the heme crevice of each of these proteins that form complementary charge-pair interactions with the lysine amino groups surrounding the heme crevice of cytochrome *c*.

It is reasonable to expect that the cytochrome *c* binding site on flavocytochrome *c*-552 will involve four or five carboxylate groups that interact with the lysine amino groups on cytochrome *c*. Millett et al. [20] have recently found that the positively charged reagent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) is highly selective for modifying carboxyl groups located in surface domains of high negative charge density. Out of over one hundred carboxyl residues on cytochrome oxidase, only four were appreciably labeled, and three of these, at residues 112, 114 and 198 of subunit II, were found to be involved in binding cytochrome *c* [20]. The mechanism involves formation of an O-acyl intermediate which then rearranges to a stable N-acylurea group as described by Timkovich [21]. EDC was also found to specifically cross-link cytochrome *c* to subunit II by forming amide linkages between complementary carboxyl and amino groups [22]. This reagent has also been used to characterize cytochrome *c* binding to the cytochrome bc_1 complex [23,24], cytochrome *c* peroxidase [25] and plastocyanin [26], and adrenodoxin binding to adrenodoxin reductase and cytochrome P-450_{scc} [27,28]. In the present study we have used EDC to study the interaction between cytochrome *c* and flavocytochrome *c*-552.

Experimental procedures

Materials

Horse heart cytochrome *c* (Type VI) and EDC were obtained from Sigma Chemical Co. A cytochrome *c* derivative in which all 19 lysine amino groups were dimethylated was prepared according to the procedure of Geren et al. [27]. *C. vinosum* flavocytochrome *c*-552 was purified by a slight modification of the method of Bartsch [29], using Sephacryl S-200 for the gel filtration steps and omitting ammonium sulfate precipitation. The resulting protein had an absorbance ratio (A_{280} : A_{410}) ranging from 0.50 to 0.60 and showed only two bands on SDS gel electrophoresis corresponding to the flavin and heme containing subunits with M_r values of 46 000 and 21 000, respectively. It was important to use highly purified enzyme preparations, since it was observed that EDC treatment of preparations with lower purity did not inhibit the sulfide : cytochrome *c* reductase activity to as great an extent.

Methods

A solution containing 15 μM flavocytochrome *c*-552 and 30 μM cytochrome *c* in 10 mM Mops (pH 6.5) was incubated with 0.2 to 10 mM EDC at room temperature. After four hours, the remaining EDC was quenched by addition of 0.1 M ammonium acetate. The sulfide : cytochrome *c* reductase activity was measured by adding an aliquot (10 nM final concentration) of the EDC-treated

flavocytochrome *c*-552 to a solution containing 5 μM cytochrome *c*, 2.5 μM sodium sulfide, and 100 mM Tris-Cl (pH 8.0) and following the reduction at 550 nm on a Cary 210 spectrophotometer. The EDC-treated flavocytochrome *c*-552 samples were then dissociated for 1 h in 4 M urea and 3% SDS at room temperature, and subjected to SDS polyacrylamide gel electrophoresis using the buffer system of Laemmli [30] with a 5% stacking gel and a 14% running gel, both containing 6 M urea. The 1.4 mm slab gels were then stained for heme with tetramethylbenzidine as described by Thomas et al. [31], and scanned at 610 nm using a Gilford scanner. The gels were then destained with sodium sulfite, restained with Coomassie brilliant blue, and scanned at 560 nm. The M_r values reported for the cross-linked dipeptides were estimated from the mobilities on SDS gels using cytochrome *c* and the native heme and flavin peptides of flavocytochrome *c*-552 as standards.

Results

Treatment of flavocytochrome *c*-552 with EDC resulted in partial loss of sulfide:cytochrome *c* reductase activity. Fig. 1 shows the effect of treatment with 0.2 to 2 mM EDC for 4 h in Mops buffer (pH 6.5) at 25°C. The percent loss in activity was significantly smaller when the reaction mixture contained 2 mol of horse heart cytochrome *c* per mole of flavoprotein, indicating that cytochrome *c* binding protected some of the carboxyl groups from modification by EDC. SDS gel electrophoresis was then used to determine whether EDC led to cross-linking between lysine amino groups on cytochrome *c* and carboxyl groups on the flavoprotein. Two bands were detected when the SDS gel of native flavocytochrome *c*-552 was stained with Coomassie blue (top left trace of Fig. 2). They are assigned to the flavin subunit with an M_r of 46000 (F) and the heme subunit with an M_r of 21000 (H). The flavin subunit was stained much more intensely by Coomassie blue than the heme subunit, and so it was necessary to increase the absorbance scale by a factor of 4 in the lower molecular weight region. Only the heme peptide was detected when the gel was stained with the tetramethyl-benzidine/H_2O_2 heme stain (top right trace of Fig. 2). Treatment of

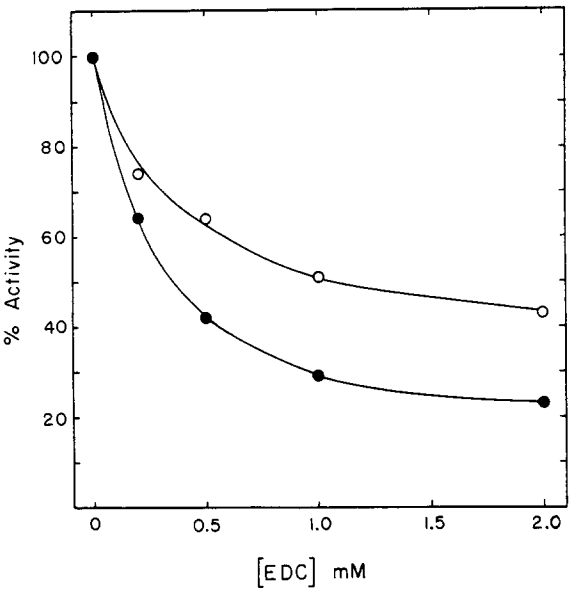

Fig. 1. Effect of EDC treatment on the sulfide:cytochrome *c* reductase activity of flavocytochrome *c*-552. 15 μM flavocytochrome *c*-552 was incubated in the presence (○) or absence (●) of 30 μM cytochrome *c* with 0 to 2.0 mM EDC in 10 mM MOPS (pH 6.5) at 25°C. After 4 h the remaining EDC was quenched by addition of 0.1 M sodium acetate. The enzyme activity was measured by adding 10 nM flavocytochrome *c*-552 to a solution containing 5 μM cytochrome *c*, 2.5 μM sodium sulfite in 100 mM Tris Cl (pH 8.0) and following the reduction at 550 nm.

flavocytochrome *c*-552 with 0.5 mM EDC for 4 h resulted in the formation of a new band with an M_r of 42000, and a decrease in intensity of the heme peptide band (middle left trace of Fig. 2). The 42000 band was stained very intensely by the heme stain (middle right trace), and was thus identified to be a cross-linked dimer of the heme peptide. The intensity of the native heme peptide band decreased to a somewhat greater extent than enzyme activity as the concentration of EDC was increased.

Significantly less heme peptide dimer was formed when cytochrome *c* was present during the EDC treatment of flavocytochrome *c*-552 (bottom trace of Fig. 2). Furthermore, two new cross-linked bands were formed with M_r values of 34000 and 59000, respectively. The 34000 band was identified to be a cross-linked complex between cytochrome *c* and the heme peptide both on the basis of its M_r value and the relative intensities of the heme and Coomassie blue stains. The 59000 band

was so intensely stained with Coomassie blue that it must contain the flavin subunit, and the heme stain indicated the presence of cytochrome *c*. The M_r value of 59000 is most consistent with identifying this band to be a 1 : 1 cross-linked complex between cytochrome *c* and the flavin subunit. Fig. 3 shows the intensities of the SDS bands as a function of the concentration of EDC used to treat a mixture of cytochrome *c* and flavocytochrome *c*-552 under the same conditions described in Fig. 1. The native heme peptide and flavin bands decreased in amplitude as the concentration of EDC used was increased, while the cross-linked bands increased. The native heme peptide had almost disappeared following reaction with 2 mM

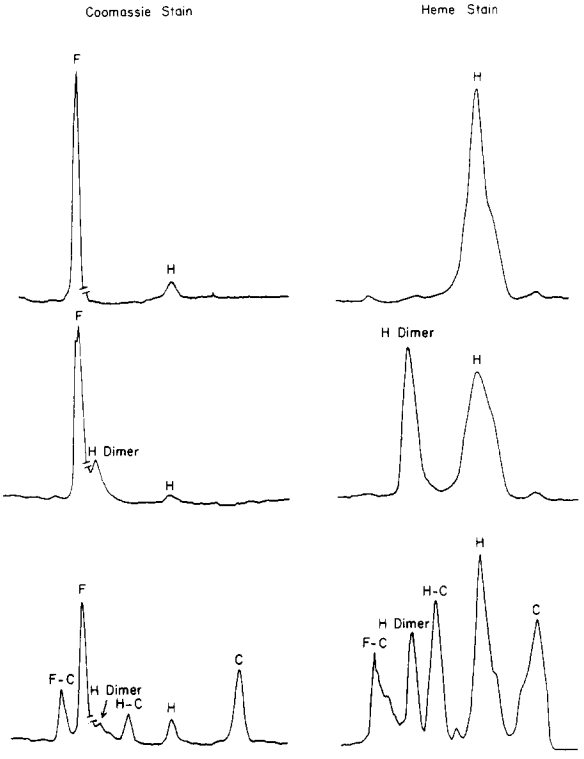

Fig. 2. SDS polyacrylamide gel electrophoresis of flavocytochrome *c*-552 treated with EDC in the presence and absence of cytochrome *c*. 15 μM flavocytochrome *c*-552 was treated with 0.5 mM EDC in 10 mM Mops (pH 6.5) for 4 h at 25°C. Aliquots containing 16 μg of the flavoprotein were subjected to electrophoresis on 14% gels, stained for heme, and scanned at 610 nm (right traces). The gels were then destained, restained with Coomassie blue, and scanned at 560 nm (left traces). Top trace: untreated flavocytochrome *c*-552; middle trace: flavocytochrome treated with EDC; lower trace: flavocytochrome treated with EDC in the presence of 30 μM cytochrome *c*

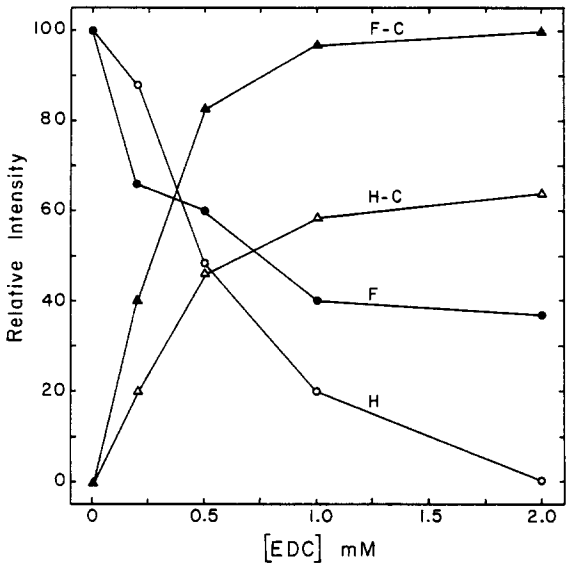

Fig. 3. The intensities of the native and cross-linked polypeptides formed upon treatment of a mixture of flavocytochrome *c*-552 and cytochrome *c* with different concentrations of carbodiimide. 15 μM flavocytochrome *c*-552 and 30 μM cytochrome *c* were treated with 0.0 to 2.0 mM EDC in 10 mM Mops (pH 6.5) for 4 h at 25°C. SDS gels were run as described in Fig. 2, and the areas under the bands were measured from scans of the Coomassie stained gels. Only relative intensities are given, since there were such large differences in the inherent staining of the different bands.

EDC, even though about 40% of the enzyme activity remained (Figs. 1 and 3). The overall cross-linking pattern was the same when only 15 μM cytochrome *c* was used during the EDC treatment. In particular, the 59000 F-C and 34000 H-C bands were formed in the same ratio at both cytochrome *c* concentrations. Formation of all of the cross-linked species was inhibited by increasing the ionic strength of the medium. Cross-linking was decreased by about 50% in the presence of 100 mM NaCl, and almost completely by 200 mM NaCl (data not shown). Experiments were also carried out using a cytochrome *c* derivative in which all of the lysines had been dimethylated, and thus protected from cross-linking. Treatment of a mixture of 15 μM flavocytochrome *c*-552 and 30 μM methylated cytochrome *c* with EDC at low ionic strength did not lead to formation of either the H-C or F-C cross-linked species. The methylated cytochrome *c* derivative did protect the enzyme from loss of sulfide : cytochrome *c* reductase

activity and formation of the heme peptide dimer, however. The dimethylated lysines retain their positive charge, and the derivative was 50% as active as native cytochrome *c* in the standard flavocytochrome *c*-552 assay.

Discussion

The loss in sulfide : cytochrome *c* reductase activity following modification of flavocytochrome *c*-552 by EDC could be the result of two different processes. EDC is known to react with carboxyl groups to form an unstable O-acylisourea which can then rearrange to form a stable N-acylurea. The conversion of a negatively charged carboxyl group at the cytochrome *c* binding site to a bulky, positively charged N-acylurea carboxyl would certainly inhibit the reaction. In addition, EDC can lead to covalent cross-linking by forming an amide bond between amino and carboxyl groups that are in close proximity. Treatment of flavocytochrome *c*-552 with EDC led to the formation of a cross-linked heme peptide dimer, indicating that the native protein exists as a dimer at the low ionic strengths used for these studies, and that carboxyl groups on one heme peptide are brought into close proximity with lysine amino groups on the other heme peptide in the dimer. There was no indication of any cross-linking between the heme peptide and the flavin peptide, or between the two flavin peptides. It was not possible to determine whether the loss in activity following EDC treatment was caused by direct modification of carboxyl groups, by intrasubunit cross-linking of adjacent lysine and amino groups, or by cross-linking the heme peptides together. The cross-linked dimer did retain some enzyme activity, since treatment with 2 mM EDC led to complete loss of the native heme peptide, but only 80% loss in enzyme activity. Gray and Knaff [4] have previously shown that flavocytochrome *c*-552 may exist as a dimer at low ionic strength, but then dissociates to the monomeric form at higher ionic strengths. Our experiments are in complete agreement with this, as the formation of the heme peptide dimer by EDC was inhibited by high ionic strength. The formation of the cross-linked heme peptide dimer was significantly decreased in the presence of cytochrome *c*. This indicates that cytochrome *c* binding is competitive with dimer formation, possibly because cytochrome *c* binds to the same site on the heme peptide as that involved in dimer formation.

Cytochrome *c* was cross-linked to both the heme peptide and the flavin peptide upon treatment with EDC. This was somewhat unexpected, since the proposed reaction mechanism involves reduction of cytochrome *c* by the heme subunit. The extent of cytochrome *c* cross-linking to the flavin subunit was about the same as that to the heme subunit, even at substoichiometric cytochrome *c* concentrations. The experiments with methylated cytochrome *c* demonstrate that both the H-C and F-C dimers are cross-linked by amide bonds between lysine amino groups on cytochrome *c* and carboxyl groups on the heme and flavin subunits. The cross-linked complex must retain some enzyme activity, since treatment of a mixture of cytochrome *c* and flavocytochrome *c*-552 with 2 mM EDC led to complete loss of the native heme peptide, but only 60% loss in enzyme activity. Gray and Knaff have previously shown that 2 mol of cytochrome *c* can bind to 1 mol flavocytochrome *c*-552, and suggested that one cytochrome *c* was binding close to each of the two heme *c* groups in the heme subunit. However, there is evidence that the two heme groups have different properties [32,33], and it is possible that the two cytochrome *c* binding sites are not identical. One binding site could be located at an interface between the heme and flavin subunits, where cytochrome *c* could be cross-linked to either subunit. If there are two different binding sites, then they must have equal affinity for cytochrome *c*, since the enzyme obeys simple Michaelis–Menten kinetics with no indication of two different phases [34]. Davidson et al. [35] have recently found that the isolated heme subunit binds to a cytochrome *c* affinity column at low ionic strength, but the flavin subunit does not. Therefore, the major contribution to cytochrome *c* binding (at both sites) is provided by the heme subunit.

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