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THE USE OF A WATER-SOLUBLE CARBODIIMIDE TO CROSS-LINK CYTOCHROME *c* TO PLASTOCYANIN

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A water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, has been used to cross-link horse heart cytochrome *c* to spinach chloroplast plastocyanin. The complex was formed in yields up to 90%, and was found to have a stoichiometry of 1 mol plastocyanin per mol cytochrome *c*. The cytochrome *c* in the complex was fully reducible by ascorbate and potassium ferrocyanide, and had a redox potential only 25 mV less than that of native cytochrome *c*. The complex was nearly completely inactive towards succinate-cytochrome *c* reductase and cytochrome *c* oxidase, suggesting that the heme crevice region of cytochrome *c* was blocked. We propose that the carbodiimide promoted the formation of amide cross-links between lysine amino groups surrounding the heme crevice of cytochrome *c* and complementary carboxyl groups on plastocyanin. It is of interest that the high-affinity site for cytochrome *c* binding on bovine heart cytochrome *c* oxidase has recently been found to involve a sequence of subunit II with some homology to the copper-binding sequence of plastocyanin.

Introduction

The electron-transport chains involved in mitochondrial oxidative phosphorylation and chloroplast photosynthesis show many remarkable similarities. Among the most completely characterized components of these chains are, respectively, mitochondrial cytochrome *c* and chloroplast plastocyanin. The very acidic blue copper protein plastocyanin shows some similarity in sequence to subunit II of mitochondrial cytochrome *c* oxidase, which has been proposed to bind one of the two copper atoms in the oxidase [1]. This is of particular interest in view of our recent finding that the high-affinity binding site for cytochrome *c* on cytochrome *c* oxidase is located at subunit II, and includes carboxyl groups close to the proposed copper-binding site [2,3]. Cytochrome *c* does rapidly reduce plastocyanin, although the rate constant, $1 \cdot 10^6\ m^{-1} \cdot s^{-1}$, is about 30-fold less than the rate constants for the physiological redox pairs cytochrome *f*-plastocyanin and cytochrome *c*-cytochrome *c* oxidase [4].

Extensive chemical modification studies have identified a group of seven or eight lysine residues on cytochrome *c* that are important for complex formation with cytochrome *c* oxidase [5–8], cytochrome c_1 [7–10], cytochrome *c* peroxidase [11,12], cytochrome b_5 [13], sulfite oxidase [14,15] and adrenodoxin [16]. These highly conserved lysines form a ring of positively charged amino groups immediately surrounding the heme crevice on the front face of cytochrome *c* [17]. X-ray crystallographic studies have located a ring of negatively

Abbreviations: TMPD, *N,N,N,N*-tetramethyl-*p*-phenylenediamine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Mops, 4-morpholinopropanesulfonic acid.

charged carboxylates surrounding the heme crevice of both cytochrome b_5 and cytochrome *c* peroxidase that is complementary to the ring of lysines on cytochrome *c* [18,19]. Chemical cross-linking studies have confirmed that the ring of carboxylates on cytochrome *c* peroxidase forms the cytochrome *c*-binding site [20,21].

The X-ray cyrstallographic structure of plastocyanin indicates that the copper is coordinated by the δ-nitrogen atoms of His-37 and His-87, and the sulfur atoms of Cys-84 and Met-92 [22]. The last three residues belong to a tight loop in the protein, with access of the copper atom to solvent blocked only by the imidazole group of His-87. Davis et al. [23] noted a prominent ring of carboxyl groups around the middle of plastocyanin, including residues 42–45, 59–61 and 68. Burkey and Gross [24] recently found that treatment of plastocyanin with the water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), in the presence of ethylenediamine modified an average of four carboxyl groups among the above residues to form positively charged aminoethylamide groups. This modification eliminated the requirement for Mg^{2+} in the electron-transfer reaction between plastocyanin and Photosystem I. It was proposed that Mg^{2+} might serve to bridge negatively charged carboxylates on native plastocyanin to carboxylates at the active site of Photosystem I. Farver et al. [25,26] recently found that binding Cr(III) to the acidic residues 42–45 of plastocyanin decreased the rate of reaction with Photosystem I, but left the reaction with cytochrome *f* unchanged. They proposed that cytochrome *f* reduced plastocyanin through the exposed imidazole group of His-87, while the reaction with Photosystem I was through a separate electron-transfer pathway involving aromatic residues leading from the copper atom to the surface of plastocyanin at residues 42–45.

In the present study we have found that the water-soluble carbodiimide EDC can cross-link lysine amino groups on cytochrome *c* to carboxyl groups on plastocyanin to form a 1 : 1 complex in high yield. The reaction conditions required for optimum cross-linking have been studied extensively. The enzymatic properties of the complex indicate that plastocyanin is cross-linked to cytochrome *c* at the heme crevice region, effectively blocking reaction with other enzymes.

Experimental Procedures

Materials. Horse heart cytochrome *c* (type VI), SDS, Tris, Mops, TMPD, sodium ascorbate and EDC were obtained from Sigma Chemical Co. Ferrous chloride, potassium chloride, hydrochloric acid, sodium phosphate, potassium ferrocyanide, ammonium sulfate, and formaldehyde, all reagent grade, were obtained from Fisher Chemical. Potassium ferricyanide, analyzed reagent, was obtained from Baker Chemical Co. DE-52 ion-exchange cellulose was obtained from Whatman Chemical Co. Bio-Rex 70, Bio-gel P-4 and P-60 were obtained from Bio-Rad Laboratories, and Sephadex G-100 was from Pharmacia. Ultrafiltration membrane cones were from Amicon and dimethylamine borane was from Aldrich.

Cytochrome *c*-depleted Keilin-Hartree particles were prepared by the method of Smith and Camerino [27] and stabilized with glycerol as described by Ferguson-Miller et al. [6]. Protein was determined by the biuret method after solubilization of the Keilin-Hartree particles as described by Jacobs et al. [28].

Plastocyanin was prepared from chloroplast membranes isolated from spinach leaves by grinding washed leaves with cold 50 mM Tris-HCl, pH 8.0, filtering through cheesecloth, and centrifuging the filtrate 40 min at $9000 \times g$ at 4°C. The precipitated membranes were resuspended in a minimum volume of 50 mM Tris-HCl, pH 8.0, and plastocyanin was isolated by a modification of the method of Ashton and Anderson [29]. Acetone which had been chilled to −20°C was added to the membranes to a concentration of 33%, and the suspension was centrifuged 20 min at $1800 \times g$ at 4°C. Chilled acetone was added to the supernatant to a final concentration of 80%, and this suspension was centrifuged 20 min at $1800 \times g$ at 4°C. The precipitate was resuspended in 50 mM Tris-HCl, pH 8.0, and dialyzed against this buffer. The dialyzed material was centrifuged 30 min at $9000 \times g$ at 4°C, and ammonium sulfate was added to the supernatant to a level of 40% saturation. After the ammonium sulfate dissolved, the suspension was centrifuged 30 min at $9000 \times g$ at 4°C. Ammonium sulfate was added to the supernatant to a final level of 80% of saturation, and the suspension was again contrifuged 30 min at $9000 \times g$ at 4°C. The supernatant was applied to a DE-52 column

at 4°C which had been equilibrated with 80% saturated ammonium sulfate in 10 mM sodium phosphate, pH 7.5. The column was washed with this buffer, and then the partially purified plastocyanin was eluted with 10 mM sodium phosphate, pH 7.5. It was applied to a second DE-52 column which had been equilibrated with this buffer, washed with the same buffer, and eluted with 50 mM potassium chloride in 25 mM sodium phosphate, pH 7.5. The impure plastocyanin was diluted with 2 vol. cold water and applied to a DE-52 column with approx. 5 ml bed volume. It was eluted from the small column with 0.5 M potassium chloride in 10 mM sodium phosphate, pH 7.5, oxidized by the addition of potassium ferricyanide, and applied to a 57×1.3 cm P-60 column which had been equilibrated with 10 mM sodium phosphate, pH 7.5. The column was eluted with this buffer, and eluant fractions which had $A_{280}/A_{600} = 1.5$ were used for further study.

Cytochrome *c* was purified on Bio-Rex 70 [5]. Both cytochrome *c* and plastocyanin were oxidized with ferricyanide and passed through a 1.0×8.0 cm. Bio-Gel P-4 column prior to use. Reduced cytochrome *c* was obtained by reaction with sodium ascorbate followed by passage through a small Bio-gel P-4 column. Methylated cytochrome *c* was produced by the reaction of dimethylamine borane and formaldehyde [30] with cytochrome *c*. The reaction was optimized using 5 mg/ml cytochrome *c* in 0.2 M sodium phosphate, pH 7.0, to which two additions of 60 mM $BH_3NH(CH_3)_2$ in methanol and 80 mM H_2CO were made at 2-h intervals for a total of 160 mM H_2CO and 120 mM $BH_3NH(CH_3)_2$. The methylated cytochrome *c* was then desalted on a small P-2 column and shown by amino acid analysis to have no more than 0.2 lysines per mol remaining unmethylated. The cytochrome *c* thus obtained retained 81.0% of its succinate-cytochrome *c* reductase activity and 17% of tis cytochrome *c* oxidase activity as measured spectroscopically [31].

Preparation of cross-linked complex. Cytochrome *c* and plastocyanin (30 μM each) were treated with 1 mM EDC at 25°C for 25 h in 5 mM Mops, pH 6.5. The reaction mixture (3–4 ml) was concentrated to 500 μl by centrifuging in Amicon Centriflo CF 25 ultrafiltration cones, and applied to a 1×70 cm G-100 column equilibrated with 100 mM sodium phosphate, pH 7.0, at 4°C. Fractions from the column were reconcentrated using the Amicon cones.

SDS-polyacrylamide electrophoresis. Samples for electrophoresis were mixed with an equal volume of buffer containing 1% SDS, 1% β-mercaptoethanol and 400 mM sodium phosphate, pH 3.0, to stop the cross-linking reaction. Electrophoresis was carried out as described by Weber and Osborn [32].

Amino acid analysis. Samples were hydrolyzed in duplicate for 22 h at 110°C with 6 M HCl in evacuated sealed tubes, and separated on a microcomputer-controlled amino acid analyzer with a ninhydrin detection system [33].

Spectral analysis. Spectra were recorded on a Cary 210 spectrophotometer. The reduction potential of the cross-linked complex was measured as described by Wada and Okunuki [34], by adding aliquots of potassium ferrocyanide to the sample in 100 mM sodium phosphate, pH 7.0, in a cuvette purged with argon.

Enzyme assays. The succinate-cytochrome *c* reductase activity of the complex was measured using Keilin-Hartree particles treated with 5% sodium deoxycholate (1 mg/mg protein) and diluted with 100 mM sodium phosphate, pH 7.0. The Keilin-Hartree particles (50 μg/ml) were then incubated for 5 min at 25°C with 5 mM sodium succinate and 1 mM potassium cyanide in 100 mM sodium phosphate, pH 7.0, to inhibit cytochrome oxidase. Then the cross-linked complex, or native ferricytochrome *c*, was added to a final concentration of 1–8 μM, and the reduction followed at 550 nm. The cytochrome *c* oxidase activity was measured with the Gilson model KM Clark electrode using the ascorbate-TMPD system. Assays were carried out at 25°C in 50 mM K^+-Mops, 200 mM sucrose, 7 mM ascorbate and 0.7 mM TMPD. The source of the cytochrome *c* oxidase was Keilin-Hartree particles treated with sodium deoxycholate (1 mg/mg protein) and added to the oxygen electrode cell to a final concentration of 70 μg protein/ml. Cytochrome *c* concentrations ranged from 0.01 to 0.35 μM.

Results

Plastocyanin is cross-linked to cytochrome c by EDC

The water-soluble carbodiimide EDC was found

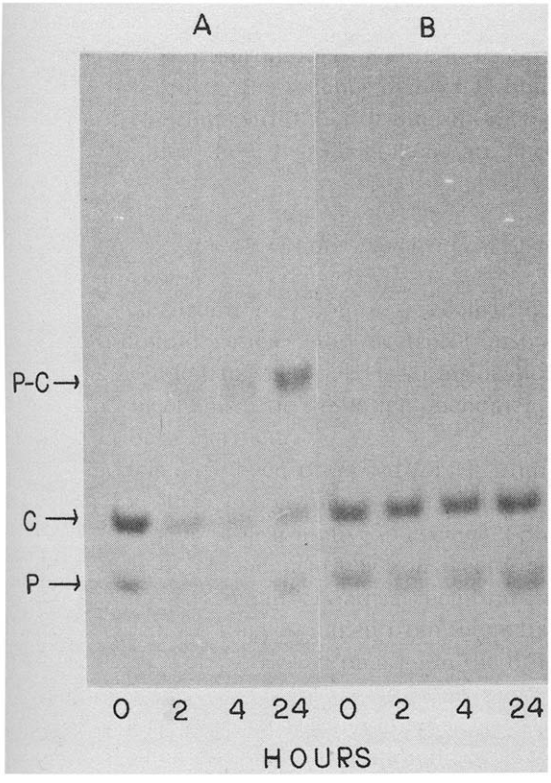

Fig. 1. SDS-polyacrylamide gel electrophoresis of plastocyanin cross-linked to cytochrome *c* (P-C) by EDC. Samples containing 81 μM plastocyanin (P) and 70 μM cytochrome *c* (C) (A), or 81 μM plastocyanin and 60 μM methylated cytochrome *c* (B), were treated with 1 mM EDC in 5 mM Mops, pH 6.5, at 25°C. 15-μl aliquots were removed at the indicated times and subjected to SDS gel electrophoresis.

to cross-link effectively plastocyanin to cytochrome *c* (Fig. 1). The complex was formed in yields of up to 90% under optimum conditions of 80 μM plastocyanin and cytochrome *c*, 1 mM EDC and 5 mM Mops at pH 6.5. It was necessary to carry out the reaction at low ionic strength in order to maximize the electrostatic interaction between the positively charged cytochrome *c* and the negatively charged plastocyanin. The cross-linking reaction was not as efficient at pH values above 7.0, while below pH 6.0 plastocyanin becomes denatured. A cytochrome *c* derivative in which all 19 lysine amino groups were dimethylated was not cross-linked to plastocyanin (Fig. 1), demonstrating that the cross-link involved the formation of amide bonds between lysine amino groups on cytochrome *c* and carboxyl groups on plastocyanin.

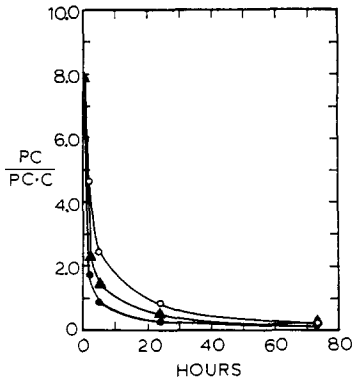

Fig. 2. The time dependence of EDC-promoted cross-linking between 37 μM plastocyanin and 84 μM cytochrome *c*. The ratio (PC/PC·C) of the SDS gel peak area of unmodified plastocyanin to the peak area of the plastocyanin-cytochrome *c* complex is plotted vs. incubation time. The reactions were carried out using 0.74 mM EDC in 5 mM Mops, pH 6.5, with ferricytochrome *c* at 25°C (●), ferrocytochrome *c* at 25°C (▲) and ferricytochrome *c* at 4°C (○).

SDS gel electrophoresis indicated that neither cytochrome *c* nor plastocyanin was appreciably polymerized when the separate proteins were treated with EDC. The yield of cross-linked complex could not be increased above 90% by carrying out the reaction with more than 1 mM EDC, or by using an excess of cytochrome *c*. The time dependence of the reaction, shown in Fig. 2, indicates that cross-linking was essentially complete after 10 h at 25°C, or after 24 h at 4°C. Ferrocytochrome *c* was cross-linked to plastocyanin somewhat more slowly than ferricytochrome *c* (Fig. 2).

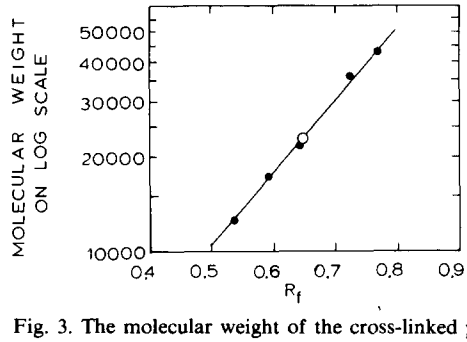

Fig. 3. The molecular weight of the cross-linked plastocyanin-cytochrome *c* complex (○) was estimated to be about 23000 by thin-layer chromatography on Sephadex superfine G-100 using a Pharmacia apparatus. The standards (●) used were: ovalbumin, soybean trypsin inhibitor, β-lactoglobulin, cytochrome *c* and myoglobin.

The stoichiometry of the cross-linked plastocyanin-cytochrome c complex is 1 : 1

The cross-linked plastocyanin-cytochrome *c* complex was purified for further study by gel chromatography on a 2 × 70 cm Sephadex G-100 column, using high ionic strength to prevent non-cross-linked plastocyanin and cytochrome *c* from migrating down the column as a complex. The purified cross-linked complex gave a single sharp band on SDS gel electrophoresis, indicating high purity. Thin-layer chromatography on Sephadex superfine G-100 was used to estimate that the molecular weight of the complex was about 23 000, consistent with a stoichiometry of 1 : 1 (Fig. 3). The amino acid composition of the complex was equal to the sum of the composition of one plastocyanin molecule and one cytochrome *c* molecule, confirming the 1 : 1 stoichiometry (data not shown).

The visible absorption spectrum of the cross-linked complex was nearly identical to that of a 1 : 1 mixture of native cytochrome *c* and plastocyanin, the major contribution from plastocyanin being above 550 nm (Fig. 4). There were no spectral shifts or other changes that would indicate a possible conformational change. At high concentrations, a 695 nm band was observed with nearly the same intensity as that of native cytochrome *c*, indicating that the Met-80 coordination to the iron was intact. It was difficult to quantitate the extinction coefficient of this band precisely, because it was at a wavelength corresponding to a minimum in the plastocyanin spectrum.

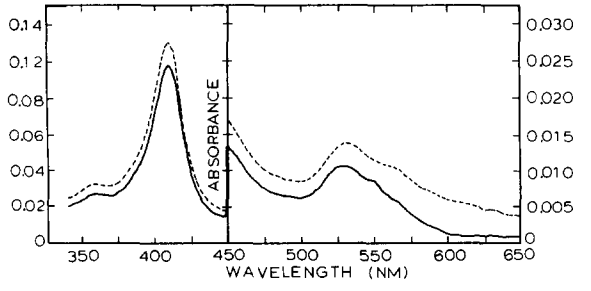

Fig. 4. The visible absorption spectrum of 1.0 μM purified cross-linked plastocyanin-cytochrome *c* complex (———) is compared to that of 0.92 μM native cytochrome *c* (- - - - -). Both samples were in 0.1 M sodium phosphate, pH 7.0, at 25°C.

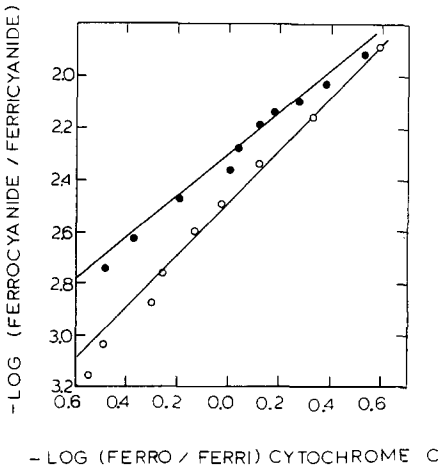

Fig. 5. Secondary plots of the ferrocyanide reduction of the plastocyanin-cytochrome *c* complex (○) and native cytochrome *c* (●). Each sample contained 3 μM cytochrome *c* in 100 mM sodium phosphate, pH 7.0, and 0–8 mM ferrocyanide. The extent of reduction of cytochrome *c* was measured at 550 nm.

The cross-linked complex reacts with small redox molecules, but not with cytochrome c oxidase or succinate-cytochrome c reductase

The cytochrome *c* in the complex was reducible with ascorbate, dithionite, or ferrocyanide, indicating that the heme crevice was not blocked to small molecules. The reaction with each of these molecules was essentially complete within a mixing time of about 30 s, but the rate constants were not measured by stopped flow. Titration with ferrocyanide was used to estimate that the redox potential of the cytochrome *c* in the complex was 235 ± 20 mV (Fig. 5) [34]. Since this is only 25 mV lower than the redox potential of native cytochrome *c*, the structure in the vicinity of the heme crevice is not too seriously perturbed by cross-linking. However, the complex had no detectable activity towards succinate-cytochrome *c* reductase, and less than 10% normal activity towards cytochrome *c* oxidase. The cytochrome oxidase activity was too low to permit a careful kinetic analysis, but it appeared that the maximum velocity was the parameter most affected.

Discussion

A wide variety of different cross-linking reagents are now available for characterizing protein-

protein interactions. Ideally, such a reagent should (a) be capable of cross-linking the type of functional groups expected to contribute to the protein-protein interaction, (b) not significantly perturb the interaction, and (c) be short enough so that the true complementary residues on each protein can be identified. The most commonly used type of reagent for characterizing the interaction of cytochrome *c* with its redox partners has been heterobifunctional arylazides [21,35]. These are generally first attached to cytochrome *c* lysines in the dark, and then photoactivated after a complex between cytochrome *c* and the redox partner is formed. A disadvantage in their use is that the photoactivated azide is quite selective for modifying nucleophilic groups such as histidine or tyrosine, which would not normally be expected to interact with lysine side chains [35]. Water-soluble carbodiimide is ideally suited for studying the interaction between cytochrome *c* and its redox partners, because it is expected to form 'zero-length' amide cross-links between lysine amino groups on cytochrome *c* and their complementary carboxyl groups on the partners [36]. This technique has recently been used to determine that the binding site for cytochrome *c* on cytochrome *c* oxidase is located at subunit II [2]. Cytochrome *c* has also been cross-linked to cytochrome *c* peroxidase by EDC, and preliminary peptide-mapping studies [20] have shown that the interaction between the two proteins is consistent with the hypothetical model proposed by Poulos and Kraut [19].

In the present study we have optimized the carbodiimide reaction conditions to cross-link specifically cytochrome *c* to plastocyanin in very high yield (90%). In previous studies with cytochrome *c* oxidase and cytochrome *c* peroxidase, the yield of cross-linked product was only 20–30%. Furthermore, the stoichiometry of the cytochrome *c*-plastocyanin complex was shown to be 1 : 1, even when one protein was in excess of the other during the reaction. Very mild conditions were used for the cross-linking reaction, and the heme environment and redox properties of cytochrome *c* were not significantly perturbed in the complex. However, the complex was almost totally inactive towards cytochrome *c* reductase and cytochrome *c* oxidase, suggesting that the heme crevice region of cytochrome *c* was blocked by cross-linked plastocyanin. Since all known redox partners of cytochrome *c* bind at the heme crevice, it is highly likely that EDC cross-linked lysine amino groups surrounding the heme crevice to carboxyl groups on plastocyanin. Although we have not identified which carboxyl groups were involved in the cross-links, they are probably among the adjacent acidic residues 42–45 and 59–61 which Burkey and Gross [24] found to be susceptible to modification by carbodiimide. These are the residues that form a ring of negative charges around the plastocyanin molecule [23], and would appear to be favorably oriented for interacting with cytochrome *c* lysines.

We have recently found that EDC specifically modified four carboxyl groups on subunit II of cytochrome oxidase, at Glu-18, Asp-112, Glu-114 and Glu-198 [3]. Residues 112, 114 and 198 were protected from modification by cytochrome *c* binding, and are therefore located at the high-affinity cytochrome *c*-binding site. The involvement of Glu-198 is particularly significant, since it is located between the two highly conserved residues Cys-196 and Cys-200 which have been proposed to ligand copper. Since this copper site is located so close to cytochrome *c*, it is probably the EPR-visible copper, and might be the initial acceptor of electrons from cytochrome *c*. If this turns out to be the case, then there might be a functional similarity between plastocyanin and subunit II of cytochrome oxidase, as well as the partial homology in the copper-binding regions of the two sequences already noted.

Acknowledgement

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