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Identification of Specific Carboxylate Groups on Cytochrome *c* Oxidase That Are Involved in Binding Cytochrome *c*†

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ABSTRACT: Modification of beef heart cytochrome *c* oxidase with 1-ethyl-3-[3-[^{14}C](trimethylamino)propyl]carbodiimide ([^{14}C]ETC) was found to dramatically inhibit the high-affinity phase of the reaction with cytochrome *c*. Reaction conditions leading to a 50% inhibition of V_{max} resulted in a 12-fold increase in the K_m for cytochrome *c*. This inhibition was accompanied by the incorporation of 1.2 ± 0.3 mol of [^{14}C]ETC into subunit II and much smaller levels of incorporation into the other subunits. The sites labeled by [^{14}C]ETC were determined by hydrolyzing subunit II with trypsin in the presence of 1% octyl β-D-glucopyranoside and separating the resulting peptides by reverse-phase high-pressure liquid chromatography. The tryptic peptides were then further hydrolyzed with *Staphylococcus aureus* protease to determine that Glu-18, Asp-112, Glu-114, and Glu-198 were the major residues labeled. Binding one molecule of cytochrome *c* to cytochrome *c* oxidase dramatically protected Asp-112, Glu-114, and Glu-198 from labeling by [^{14}C]ETC and prevented the loss in electron-transfer activity. We propose that the negatively charged carboxylates on Asp-112, Glu-114, and Glu-198 are involved in cytochrome *c* binding and that their conversion to bulky, positively charged ETC-carboxyl groups inhibits the reaction with cytochrome *c*. Asp-112 and Glu-114 are located in a highly conserved sequence (104-115) containing alternating acidic and aromatic residues. These aromatic residues could serve as an electron-transfer pathway from cytochrome *c* to cytochrome *a* or the EPR-visible copper. Glu-198 is located between the conserved cysteines-196 and -200 which have been proposed to serve as ligands for the EPR-visible copper.

The electron-transfer reaction between cytochrome *c* and cytochrome *c* oxidase is generally thought to involve the formation of a 1:1 complex stabilized by electrostatic interactions. Extensive chemical modification studies have shown that seven or eight highly conserved lysine residues immediately surrounding the heme crevice of cytochrome *c* are involved in the interaction with cytochrome *c* oxidase (Smith et al., 1977; Ferguson-Miller et al., 1978; Rieder & Bosshard, 1980), cytochrome c_1 (Ahmed et al., 1978), cytochrome *c* peroxidase (Kang et al., 1978; Smith & Millett, 1980), and cytochrome b_5 (Stonehuerner et al., 1979). X-ray crystallographic studies have identified a ring of negatively charged carboxylates surrounding the heme crevice of both cytochrome b_5 and cytochrome *c* peroxidase that is complementary to the ring of positively charged lysine residues on cytochrome *c* (Salemme, 1976; Poulos & Kraut, 1980). Recent cross-linking studies have supported the proposal that the ring of carboxylates on cytochrome *c* peroxidase forms the cytochrome *c* binding site (Bisson & Capaldi, 1981; Waldmeyer et al., 1982).

It is reasonable to expect that the cytochrome *c* binding site on cytochrome *c* oxidase would also involve a complementary ring of carboxylate residues. Recent attempts to localize the cytochrome *c* binding site have not been designed to identify specific carboxylate residues but have instead focused on which subunits of cytochrome *c* oxidase are involved. Arylazidolysine-13 cytochrome *c* was found to specifically cross-link to subunit II and block the high-affinity binding site for cytochrome *c* (Bisson et al., 1980). The major site of insertion of the photoactivated azide was found to be His-161 (Bisson et al., 1982a). In contrast, yeast cytochrome *c* modified at Cys-107 on the backside of the protein was cross-linked to subunit III of cytochrome *c* oxidase from both yeast (Moreland & Dockter, 1981) and beef heart (Fuller et al., 1981). On the basis of the above results, we have proposed (Capaldi et al., 1982) that cytochrome *c* binds at a cleft formed at the interface between two monomers of the cytochrome oxidase dimer (Fuller et al., 1979). The ring of lysines surrounding the heme crevice at the front of cytochrome *c* would interact with subunit II of one monomer, while the backside would be close to subunit III on the other monomer.

In the present study, we have used the reagent 1-ethyl-3-[3-[^{14}C](trimethylamino)propyl]carbodiimide ([^{14}C]ETC)[1] to identify specific carboxylate groups on cytochrome *c* oxidase that are involved in cytochrome *c* binding. We previously found that modification of cytochrome *c* oxidase with [^{14}C]-ETC resulted in inhibition of the high-affinity phase of the reaction with cytochrome *c* and incorporation of ^{14}C into subunit II (Millett et al., 1982). Equimolar concentrations of cytochrome *c* dramatically protected cytochrome *c* oxidase from both the inhibition of electron-transfer activity and the

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[1] Abbreviations: [^{14}C]ETC, 1-ethyl-3-[3-[^{14}C](trimethylamino)-propyl]carbodiimide iodide; $NaDodSO_4$, sodium dodecyl sulfate; HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; EPR, electron paramagnetic resonance.

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modification of subunit II. It was also found that cytochrome *c* was specifically cross-linked to subunit II by ETC.

In this paper, we demonstrate that [^{14}C]ETC covalently labels four specific carboxyl groups on subunit II of cytochrome *c* oxidase. Fragmentation studies identified these labeled carboxyls to be at residues Glu-18, Asp-112, Glu-114, and Glu-198. The carboxyl groups on residues 112, 114, and 198 were protected from modification by binding one molecule of cytochrome *c* to cytochrome *c* oxidase and are thus involved in the high-affinity cytochrome *c* binding site. The mechanism for the modification of these carboxyl groups by ETC probably involves the formation of an *O*-acylisourea intermediate, which then rearranges to a stable *N*-acylurea group as described by Timkovick (1977).

Experimental Procedures

Materials. Beef heart cytochrome *c* oxidase was prepared according to Capaldi & Hayashi (1972) and dialyzed for 24 h against 10 mM sodium phosphate, pH 7.0, and 1% Triton X-100 to remove sodium cholate and salts. The oxidase had a heme a content of 9.0–11.0 nmol/mg of protein and contained about 100 μg of phospholipid/mg of protein. Horse heart cytochrome *c* was obtained from Sigma. [^{14}C]ETC was synthesized according to the procedure of Sheenan et al. (1961) by treating free base EDC with [^{14}C]CH_3I (53 mCi/mmol; Research Products International Corp.). The product was diluted with unlabeled ETC to a specific activity of 10 mCi/mmol.

Reaction of Cytochrome c Oxidase with [^{14}C]ETC. Beef heart cytochrome *c* oxidase (100 μM aa_3) in 2 mL of 10 mM phosphate and 1% Triton X-100 was treated with 4 mM [^{14}C]ETC at room temperature for 12 h. The preparation was then passed through a 2 × 10 cm P-2 column equilibrated with 50 mM sodium phosphate, pH 7.0, and 1% Triton X-100 to remove the reagent. The cytochrome *c* oxidase activity was measured by following the oxidation of 1–30 μM ferrocytochrome *c* at 550 nm in 50 mM sodium phosphate, pH 7.4, containing 0.3% Tween 80. The concentration of cytochrome *c* oxidase in the assay was 10–30 nM. The first-order rate constant was calculated from a least-squares analysis of a log plot of absorbance vs. time. The rate constants were compared to those of a control treated in an identical fashion except for the [^{14}C]ETC.

Separation of Cytochrome c Oxidase Subunits. The modified cytochrome *c* oxidase was dissociated for 1 h in 5% $NaDodSO_4$, 1% β-mercaptoethanol, and 5 mM EDTA. The sample (3 mL) was then applied to a 3 × 40 cm Bio-Rad P-100 column (–400 mesh) equilibrated with 3% $NaDodSO_4$ and 10 μM dithioerythritol. Each fraction was analyzed for protein at 280 nm and for ^{14}C by mixing 5 μL with 2.5 mL of Omnifluor (New England Nuclear; 2.66 g/L in 2:1 toluene:Triton X-100) and counting on a Packard scintillation counter. The fractions containing the subunits were pooled, passed through a 5 × 20 cm Bio-Rad P-4 column to remove most of the $NaDodSO_4$, and lyophilized.

HPLC Separation of Tryptic Peptides of Subunit II. Purified subunit II was adjusted to a protein concentration of 2 mg/mL in 0.1% $NaDodSO_4$, 1% octyl glucoside, and 10 mM $NaHCO_3$, pH 8.0. TPCK-treated trypsin (Worthington, Inc) was then added to a final concentration of 0.18 mg/mL and the mixture incubated at 37 °C for 3 h. The sample was then injected into an Altex 334 HPLC with a Brownlee RP-300 column and an Altex 155-10 detector set at 210 nm. The peptides were eluted with a linear gradient going from 5 mM sodium phosphate, pH 7.0, to 25% 1-propanol in 100 min, followed by a linear gradient to 55% propanol in 60 min. The eluent was collected in 0.8-mL fractions, and 5% of each fraction was analyzed for ^{14}C. The amino acid composition of each purified peptide was determined by hydrolyzing the sample in 6 M HCl at 110 °C for 24 h in an evacuated, sealed tube. The hydrolysates were injected into a Dionex microbore amino acid analyzer equipped with ninhydrin detection. The peptides were identified by comparing the amino acid composition with the sequence of beef subunit II (Steffens & Buse, 1979).

HPLC Separation of Staphylococcus aureus Protease Digested Peptides. The purified tryptic peptide was concentrated under a stream of nitrogen to 2–4 mg of protein/mL in 50 mM sodium phosphate, pH 7.0. *S. aureus* protease (V8) (Miles Laboratories) was then added to a concentration of 50–100 μg of protein/mL, and the digestion was carried out for 12 h at 37 °C. In the case of the $T_{179-217}$ and T_{1-98} peptides, 1% octyl glucoside was also included in the digestion medium. The T_{99-134} and T_{1-98} peptide digests were separated on the RP-300 column by using a gradient from 5 mM sodium phosphate, pH 7.0, to 1-propanol, while the $T_{179-217}$ digests were separated by using a gradient from 0.1% trifluoroacetic acid to 1-propanol. The amino acid composition and ^{14}C content of each purified peptide were determined as previously described.

Table I: Effect of Cytochrome *c* Binding on the Incorporation of [^{14}C]ETC into Subunit II of Cytochrome *c* Oxidase[a]

sample modified with [^{14}C]ETC	cyt aa_3	cyt aa_3–cyt *c* complex
V_{max} (% native)	50	90
$K_m/[K_m$(native)]	12	3
[^{14}C]ETC incorpd into subunit II	395 (1.2)	182 (0.5)
[^{14}C]ETC incorpd into residues 1–98	83 (0.25)	83 (0.25)
[^{14}C]ETC incorpd into residues 99–134	216 (0.66)	58 (0.18)
[^{14}C]ETC incorpd into residues 179–227	85 (0.26)	35 (0.11)

[a] Cytochrome *c* oxidase in the presence or absence of cytochrome *c* was modified with [^{14}C]ETC and separated into its subunits as described in Figure 1. Subunit II was digested with trypsin, and the resulting peptides were separated by HPLC as described in Figure 2. The radioactivities are given in units of 10^3 cpm. The numbers in parentheses are the moles of [^{14}C]ETC incorporated per mole of subunit II.

Results

Cytochrome c Protection of Subunit II from Modification by [^{14}C]ETC. The modification of cytochrome *c* oxidase by [^{14}C]ETC was found to significantly inhibit the high-affinity phase of the reaction with cytochrome *c*, as previously described (Millett et al., 1982). Under conditions which led to 50% inhibition of V_{max}, the K_m for cytochrome *c* was increased 12-fold (Table I). Separation of [^{14}C]ETC-labeled cytochrome *c* oxidase into its component subunits by chromatography on P-100 revealed that subunit II was the major site of radiolabeling, with 1.2 ± 0.3 mol of [^{14}C]ETC incorporated per mol of subunit II (Figure 1). Equimolar concentrations of cytochrome *c* dramatically protected cytochrome *c* oxidase from inhibition by the carbodiimide and incorporation of [^{14}C]ETC into subunit II (Figure 1, Table I). The only other polypeptide that cytochrome *c* protected from modification by [^{14}C]ETC was component b or c, in agreement with our previous studies (Millett et al., 1982).

Separation of Subunit II Tryptic Peptides by Reverse-Phase HPLC. It has not previously been possible to completely hydrolyze subunit II into its component peptides with trypsin.

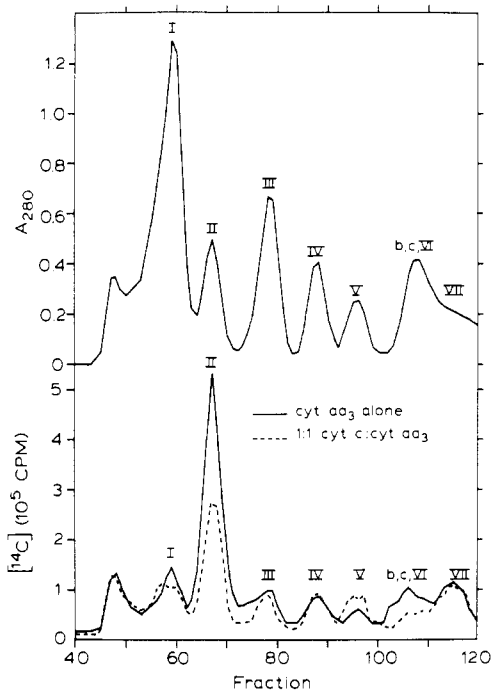

FIGURE 1: Effect of cytochrome *c* binding on the incorporation of [^{14}C]ETC into cytochrome *c* oxidase subunits. Cytochrome *c* oxidase (115 μM) with or without 115 μM cytochrome *c* was treated with 4 mM [^{14}C]ETC for 12 h in 10 mM phosphate, pH 7.0, and 1% Triton X-100. The oxidase subunits were separated on a 3 × 40 cm Bio-Rad P-100 column eluted with 3% $NaDodSO_4$. The A_{280} trace at the top of the figure is for the sample treated in the absence of cytochrome *c*. The A_{280} trace for the sample with cytochrome *c* was essentially the same except for extra absorbance due to cytochrome *c* coeluting with subunit V. The traces at the bottom of the figure show the incorporation of [^{14}C]ETC. The fraction size was 1.35 mL.

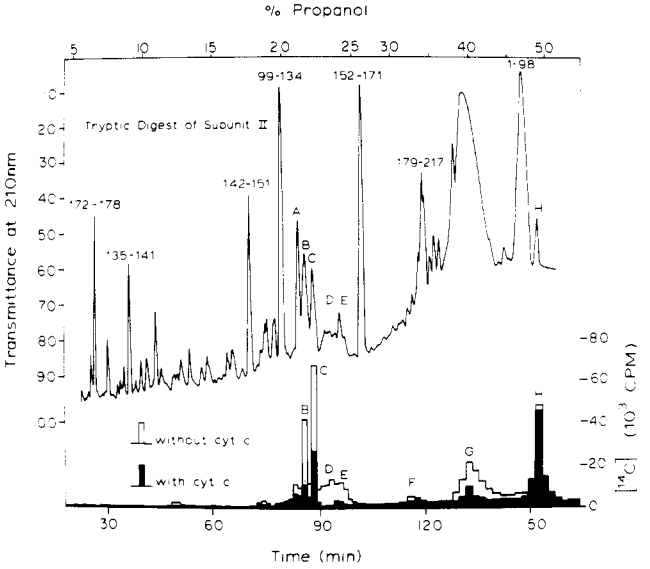

FIGURE 2: Separation of tryptic peptides of [^{14}C]ETC-labeled subunit II by reverse-phase HPLC. Purified [^{14}C]ETC-labeled subunit II (0.6 mg) was digested with trypsin and eluted on a Brownlee RP-300 column with a linear gradient from 5 mM phosphate, pH 7.0, to 25% 1-propanol in 100 min, followed by a linear gradient to 55% propanol in 60 min. The flow rate was 0.8 mL/min. The A_{210} trace at the top of the figure is for the sample treated in the absence of cytochrome *c*. The A_{210} trace of the sample treated in the presence of cytochrome *c* was the same except that peaks A–E were smaller. The histogram at the bottom of the figure shows [^{14}C]ETC incorporation in the presence and absence of cytochrome *c*.

Varying amounts of intact subunit II and large concatamers remained after extensive hydrolysis (Bisson et al., 1982b). We have overcome this problem by including 1% octyl glucoside in the digestion medium to optimally solubilize subunit II without denaturing trypsin. $NaDodSO_4$ gel electrophoresis revealed that no undigested subunit II remained after treatment with 8% trypsin for 3 h at 37 °C in 10 mM $NaHCO_3$, pH 8.0, containing 1% octyl glucoside and 0.16% $NaDodSO_4$ (F. A. Marshall and R. A. Capaldi, unpublished results). The subunit II tryptic peptides were separated with much higher resolution than previously possible by using reverse-phase HPLC on a Brownlee RP-300 column with a gradient from 5 mM sodium phosphate, pH 7.0, to 1-propanol. The tryptic chromatogram of native subunit II was nearly identical with that of [^{14}C]ETC-labeled subunit II shown in Figure 2 except that the derivative peptides labeled A–H were totally absent. The seven major tryptic peptides from native subunit II were identified by amino acid analysis and are indicated by the sequence numbers shown in Figure 2 and Table II. These peptides were eluted in the order of increasing hydrophobicity, with the hydrophilic peptide $T_{172-178}$ eluting at a low concentration of propanol and the very hydrophobic, membrane-spanning peptide T_{1-98} not eluting until 46% propanol. The entire chromatogram was extremely reproducible from one run to the next, except that the two small peaks immediately following $T_{179-217}$ were often larger relative to $T_{179-217}$ than shown in Figure 2. These peptides were identified as $T_{179-221}$ and $T_{179-227}$, indicating that the peptide bonds involving lysines-217 and -221 were incompletely hydrolyzed under these conditions. If the trypsin digestion was allowed to proceed for longer times, the peptide bonds involving Lys-49, Arg-82, Lys-217, and Lys-221 were more completely hydrolyzed. This

Table II: Primary Sequence of Subunit II from Beef Cytochrome *c* Oxidase[a]

M	A	Y	P	M	Q	L	G	F	Q	D	A	T	S	P	I	M	E*	E	L	L	H	F	H	D	H	T	L	M	I
1									10										20												30
V	F	L	I	S	S	L	V	L	Y	I	I	S	L	M	L	T	T	K	L	T	H	T	S	T	M	D	A	Q	E
									40										50												60
V	E	T	I	W	T	I	L	P	A	I	I	L	I	L	I	A	L	P	S	L	R	I	L	Y	M	M	D	E	I
									70										80												90
N	N	P	S	L	T	V	K	T	M	G	H	Q	W	Y	W	S	Y	E*	Y	T	D*	Y	E	D	L	S	F	D	S
									100										110												120
Y	M	I	P	T	S	E	L	K	P	G	E	L	R	L	L	E	V	D	N	R	V	V	L	P	M	E	M	T	I
									130										140												150
R	M	L	V	S	S	E	D	V	L	H	S	W	A	V	P	S	L	G	L	K	T	D	A	I	P	G	R	L	N
									160										170												180
Q	T	T	L	M	S	S	R	P	G	L	Y	Y	G	Q	C	S	E*	I	C	G	S	N	H	S	F	M	P	I	V
									190										200												210
L	E	L	V	P	L	K	Y	F	E	K	W	S	A	S	M	L															
									220										227												

[a] From Steffens & Buse (1979). The peptide bonds hydrolyzed by trypsin are indicated, and the residues labeled by [^{14}C]ETC are starred.

was not desirable, however, because the [^{14}C]ETC-labeled carboxyl groups were somewhat labile under the alkaline conditions used for the tryptic hydrolysis. The seven major peptides shown in Figure 2 plus $T_{179-221}$ and $T_{179-227}$ account for the entire sequence of native subunit II. The very broad peak eluting at 40% propanol and the sharp peak on its leading edge are impurities or artifacts due to gradient mixing, since they were observed in the absence of sample. They could not be removed by redistillation of the 1-propanol or the water used in the buffers.

The tryptic chromatogram of [^{14}C]ETC-modified subunit II shown in Figure 2 contains all the peptides found in native

subunit II, plus eight new peptides labeled A–H. The barograph shown at the bottom of Figure 2 indicates that peptides B–H were radiolabeled, while peptide A was not, and must therefore contain some type of internal cross-link. Peptides A–E were all found to have the same amino acid composition as native peptide T_{99-134}. Amino acid analysis indicated that peptide G was a [^{14}C]ETC-labeled mixture of $T_{179-217}$, and $T_{179-226}$. It could not be detected at 210 nm because it eluted at the same position as the broad artifact. Amino acid analysis showed that there were no peptides present in this region of the tryptic chromatogram of native subunit II. Peptide H was identified by amino acid analysis to be a labeled form of the native peptide T_{1-98}. In every case, the modified peptides eluted at higher propanol concentrations than the corresponding native peptide because of the replacement of a carboxylate group with a more hydrophobic ETC-carboxyl group. Equimolar concentrations of cytochrome *c* were found to protect cytochrome *c* oxidase from modification by [^{14}C]ETC and inhibit the formation of peptides A–E and G (Figure 2, Table I). This protection was in approximately the same proportion as the protection of electron-transfer activity (Table I). The modification of T_{1-98} to form peptide H was not affected by cytochrome *c* and served as an internal control on our experimental design.

We also carried out experiments in which cytochrome *c* oxidase was labeled much more extensively by using 14 mM [^{14}C]ETC. Under these conditions, the electron-transfer activity (V_{max}) was 98% inhibited, and 4.8 [^{14}C]ETC groups were incorporated into subunit II. All of the derivative peptides A–H were produced in larger amounts, but there was only one additional peptide observed, eluting right after peptide H. This was identified as a doubly labeled form of T_{1-98}. Furthermore, the native peptides T_{99-134} and $T_{179-217}$ were totally missing, indicating that these peptides were completely modified under these conditions. When the modification was carried out with 14 mM [^{14}C]ETC in the presence of equimolar cytochrome *c*, the electron-transfer activity was 75% inhibited, and native peptides T_{99-134} and $T_{179-217}$ were present in about 25% of the amount observed in the tryptic chromatogram of unmodified subunit II. The modified peptides A–E and G were present in lower amounts than in the unprotected experiment. The tryptic hydrolysis experiments have thus demonstrated that all of the modifications of subunit II caused by [^{14}C]ETC are located in sequences 1–134 and 179–221. Even in the heavy labeling experiments, the native peptides $T_{172-178}$, $T_{135-141}$, $T_{142-151}$, and $T_{152-171}$ were not decreased, and no radiolabeled peptides were observed that could be attributed to them. There was no detectable hydrolysis of the [^{14}C]ETC-carboxyl groups under the conditions used for the tryptic hydrolysis. However, if the hydrolysis was allowed to proceed for 12 h or longer, up to 20% of the ^{14}C eluted early in the chromatogram as the free ethyl-3-[3-[^{14}C](trimethylamino)propyl]urea.

Identification of Specific Carboxyl Groups in Residues 99–134 of Subunit II That Are Labeled by [^{14}C]ETC. We found *S. aureus* protease to be uniquely suited to the further degradation of the tryptic peptides because in phosphate buffer it hydrolyzes peptide bonds to the carbonyl side of Asp and Glu residues (Drapeau, 1977). Modification of the Glu or Asp residue prevents the hydrolysis of the peptide bond and thus significantly changes the peptide fragmentation pattern. Figure 3 shows the HPLC chromatograms of *S. aureus* protease digested native T_{99-134} and the derivative peptides A, B, and C. The peptides from native T_{99-134} were identified by amino acid analysis as indicated by the lower case letters referring to the sequence at the top of the figure. The peak

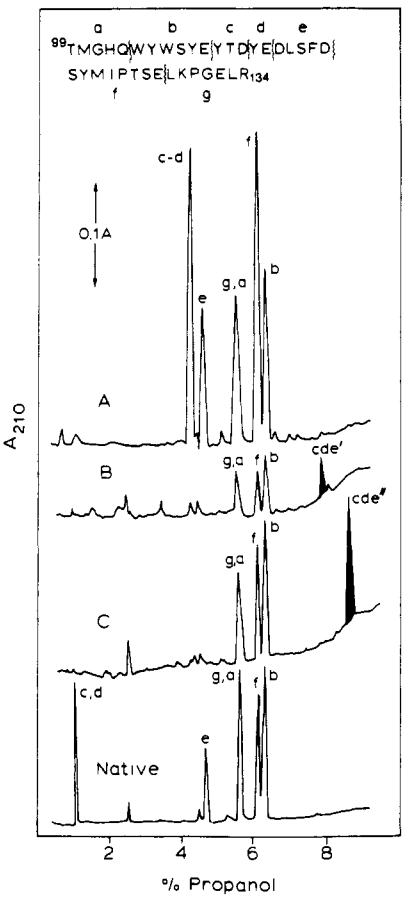

FIGURE 3: HPLC chromatograms of *S. aureus* protease digested native T_{99-134} (bottom trace), and derivative peptides A, B, and C (traces A, B, and C). The purified tryptic peptide was digested with *S. aureus* protease and eluted on a Brownlee RP-300 column with a linear gradient from 5 mM phosphate, pH 7.0, to 20% 1-propanol in 120 min. The flow rate was 0.8 mL/min. The lower case letters identifying the peaks refer to the sequence 99–134 of subunit II at the top of the figure. All of the ^{14}C radioactivity in peptides B and C eluted with cde′ and cde″, shaded in black.

marked c,d was found to have the amino acid composition of a mixture of peptide c (YTD) and d (YE). Since it eluted so early in the chromatogram, it was too hydrophilic to be a concatamer c-d. Peaks e and f were found to have the amino acid composition of the pure peptides e and f, but the peak marked g,a could only be interpreted as a 60:40 mixture of peptide g and peptide a. Since a pure peptide b was also found, *S. aureus* protease must have cleaved the peptide bond following glutamine-103, apparently because this residue was deamidinated. Cleavage did not normally occur after Asp-115 or Glu-132. The *S. aureus* protease chromatogram of peptide A (trace A in Figure 3) was the same as that of native T_{99-134} except that the two native peptides c and d were missing and a new peptide appeared with the amino acid composition of c-d. Since this peptide was not radiolabeled, it must contain some type of internal cross-link between the carboxyl group of Asp-112 and a nucleophilic group in c-d. Riehm & Scheraga (1966) proposed that a backbone NH group might react with the intermediate *O*-acylisourea to form an imide, but it also seems possible that the phenolic group of one of the neighboring tyrosines might have served as the nucleophile. The *S. aureus* protease chromatograms of peptides B and C were the same as that of native T_{99-134}, except that the native peptides c, d, and e were missing and each had one new radiolabeled peak with the amino acid composition c-d-e. The new peptide cde′ from B contained 1 equiv of ^{14}C, presumably

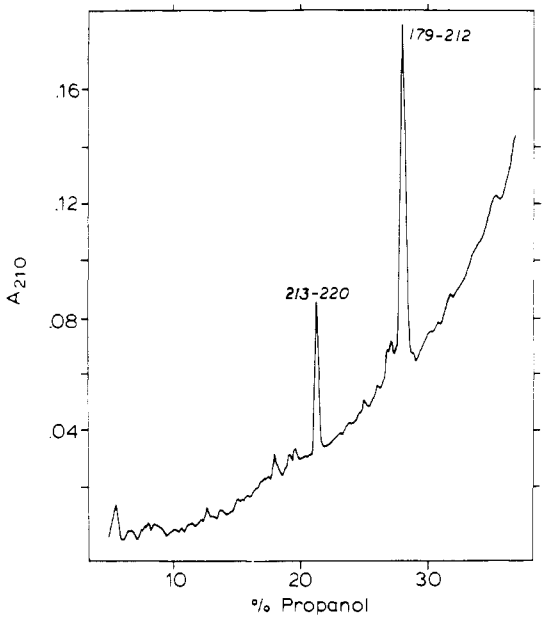

FIGURE 4: HPLC chromatograms of *S. aureus* protease digested peptide G. The tryptic peptide G purified as shown in Figure 2 was digested with *S. aureus* protease and eluted on a Brownlee RP-300 column with a linear gradient from 0.1% trifluoroacetic acid to 45% propanol in 90 min. The flow rate was 0.8 mL/min. Most of the ^{14}C radioactivity was eluted with the peptide identified as 179–212.

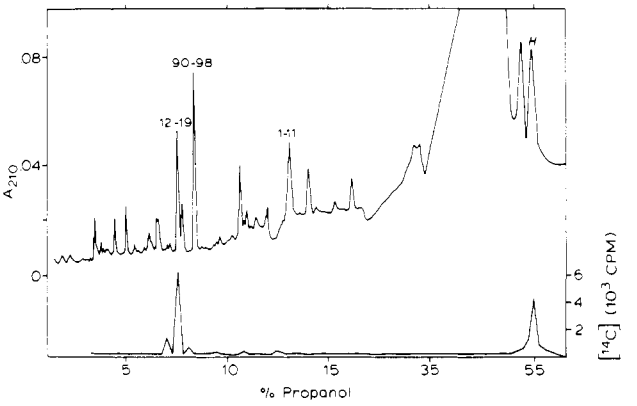

FIGURE 5: HPLC chromatogram of *S. aureus* protease digested peptide H. The digested peptide was eluted on a Brownlee RP-300 column with a linear gradient from 5 mM phosphate to 15% propanol in 90 min followed by a linear gradient to 60% propanol in 60 min.

as an *N*-acylurea attached to the carboxyl group of Glu-114. Peptide cde′ must also have an internal cross-link involving Asp-112 to explain the lack of cleavage after this residue. The new peptide cde″ from C contained 2 equiv of ^{14}C, presumably as *N*-acylurea groups at both Asp-112 and Glu-114. The *S. aureus* chromatographs of peptides D and E (not shown) were similar to those of B and C, respectively, except that they indicated an additional modification, in peptide b. Peptides D and E were radiolabeled to the same extent as peptides B and C, so the additional modification must involve some type of internal cross-link. The *S. aureus* protease fragmentation studies described above indicate that the sequence 104–114 of subunit II is highly susceptible to modification by [^{14}C]ETC, and the primary sites of modification are Asp-112 and Glu-114. Furthermore, all of these sites are protected from modification in the presence of cytochrome *c*.

Identification of Specific Carboxyl Groups Modified by [^{14}C]ETC in Residues 1–98 and 179–227 of Subunit II. The derivative peptide G found in the tryptic hydrolysate of [^{14}C]ETC-labeled subunit II was shown to be a mixture of $T_{179-211}$, $T_{179-221}$, and $T_{179-227}$. Figure 4 shows the chromatogram of a sample of G hydrolyzed with *S. aureus* protease. The two major peaks were identified by amino acid analysis to be peptides 179–212 and 213–221, indicating that this sample of G was primarily $T_{179-221}$. Most of the radioactivity eluted with peptide 179–212, which contained 1 equiv of [^{14}C]ETC per mol. Therefore, Glu-198 is most likely the site of carbodiimide labeling. There is a remote possibility that [^{14}C]ETC could have labeled a tyrosine at residues 192 or 193, instead of the carboxyl group at Glu-198 (Carraway & Koshland, 1968). However, carbodiimide modification of tyrosine has never been observed under the very mild conditions used in the present study. The binding of cytochrome *c* protected Glu-198 from modification by ETC.

The *S. aureus* chromatogram of peptide H is shown in Figure 5. The chromatogram was almost identical with that of native T_{1-98}, except for the presence of two new peptides which were each radiolabeled with 1 equiv of [^{14}C]ETC. Amino acid analysis of the peak eluting at 7.5% propanol indicated that it was peptide 12–19, and therefore must have been labeled at Glu-18. The other peptide eluted at the same position as the original peptide H and had an amino acid composition similar to, but not identical with, that of H. It could therefore be either undigested H or a partially digested peptide containing a different labeled carboxylate adjacent to one of the very hydrophobic sequences in residues 1–98. The modification of T_{1-98} was not protected in the presence of equimolar cytochrome *c*.

Discussion

The hypothetical models for the interaction of cytochrome *c* with cytochrome b_5 (Salemme, 1976) and cytochrome *c* peroxidase (Poulos & Kraut, 1980) lead us to expect a ring of five or more carboxylates at the cytochrome *c* binding site on cytochrome *c* oxidase. The electrostatic interaction between cytochrome *c* and its redox partners has been quantitatively described in a semiempirical relationship recently developed by Smith et al. (1981). This relationship, which is based on a correlation of chemical modification and ionic strength dependence data, predicts that there are six to eight specific complementary charge-pair interactions between lysine amino groups on cytochrome *c* and carboxylate groups on cytochrome *c* oxidase. It is reasonable to expect that the carboxylates involved in cytochrome *c* binding are to be found among the 11 acidic residues conserved in sequences of subunit II from five different species: beef (Steffens & Buse, 1979), human (Barrell et al., 1979), mouse (Bibb et al., 1981), yeast (Fox, 1979), and maize (Fox & Leaver, 1981). These residues are circled in the schematic folding pattern for subunit II shown in Figure 6. The hydrophobic sequences 27–48 and 63–82 are shown as α helices traversing the phospholipid bilayer, thus placing residues 1–26 and 82–227 on the cytoplasmic side of the membrane (Bisson et al., 1982b).

In order to identify which carboxylates on cytochrome *c* oxidase are involved in cytochrome *c* binding, we have utilized the water-soluble carbodiimide [^{14}C]ETC (Timkovich, 1977). This reagent was found to be highly selective in its modification of carboxyl groups at pH 7.0. Out of a total of 25 acidic residues on subunit II, only four, Glu-18, Asp-112, Glu-114, and Glu-198, were significantly labeled. Three of these groups are adjacent to other acidic residues in the sequence of subunit II, suggesting that the positively charged carbodiimide preferentially modifies carboxyl groups located in negatively charged clusters. Cytochrome *c* binding protected Asp-112, Glu-114, and Glu-198 from modification by [^{14}C]ETC and

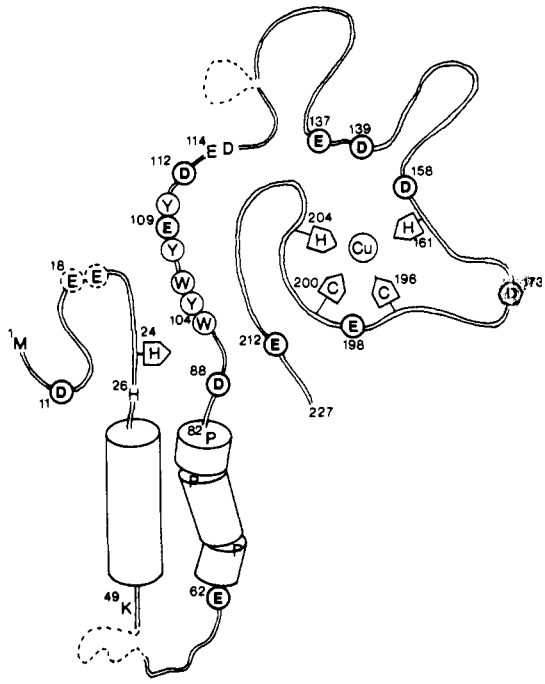

FIGURE 6: Schematic diagram of a possible folding pattern of subunit II of cytochrome *c* oxidase. The hydrophobic sequences 27–48 and 63–82 are shown as α helices traversing the phospholipid bilayer, placing residues 1–26 and 83–227 on the cytoplasmic side of the membrane. All Asp and Glu residues fully conserved in subunit II from beef, human, mouse, yeast, and maize cytochrome *c* oxidase are circled. Also shown are Glu-18 and Glu-19, conserved in three of the above species, and Glu-114 and Asp-115, which were labeled by [^{14}C]ETC. The conserved residues in the sequence 104–115 are also shown. Potential copper binding residues, Cys and His, that are fully conserved are enclosed by a pentagon. The dashed lines indicate extra residues inserted into subunit II from yeast. The figure uses the standard one-letter code for amino acids.

the resulting loss in electron-transfer activity. The conversion of negatively charged carboxylates at these residues to bulky, positively charged *N*-acylurea groups would account for the inhibition of the reaction with cytochrome *c*. Under conditions which led to the incorporation of 1.0 mol of [^{14}C]ETC per mol of these three residues, V_{max} was inhibited by 50%, and the K_m for cytochrome *c* was increased 12-fold (Table I). No other carboxylates on cytochrome *c* oxidase were protected from modification by cytochrome *c* binding except for one in component b or c. These components, however, can be removed from cytochrome *c* oxidase without loss of activity (F. Malatesta and R. A. Capaldi, unpublished results).

Our finding that Asp-112, Glu-114, and Glu-198 are all located at the high-affinity binding site for cytochrome *c* places certain constraints on the potential folding pattern for subunit II shown in Figure 6. These residues should all be on one surface of the protein within about 20 Å of each other, since this is the diameter of the binding site on cytochrome *c*. Since Glu-18 is definitely not involved in cytochrome *c* binding, the N-terminal region of subunit II need not interact directly with the C-terminal region. Glu-109 and Asp-115 are adjacent to Asp-112 and Glu-114, so they are also potential candidates for interaction with cytochrome *c* lysines. In fact, lysine residues 13, 72, 86, and 87 on cytochrome *c* form a cluster as close to one another as these four residues do on subunit II. The finding that His-161 is the site of cross-linking by arylazidolysine-13 cytochrome *c* suggests that the adjacent residues Glu-157 and Asp-158 might also be involved in cytochrome *c* binding (Bisson et al., 1982a). This brings the number of carboxylate groups that are potentially involved in cytochrome *c* binding to 7, e.g., at residues 109, 112, 114, 115, 157, 158, and 198.

A number of lines of evidence suggest that at least one of the copper atoms in cytochrome *c* oxidase is located in subunit II. Winters et al. (1980) found that both heme and copper were associated exclusively with subunits I and II during electrophoresis under mild denaturing conditions. *Paracoccus denitrificans* cytochrome *c* oxidase contains two coppers and two heme *a* groups but only two subunits, which are immunologically cross-reactive with subunits I and II of the eukaryotic enzyme (Ludwig, 1980). Recent EPR and EXAFS studies indicate that the EPR-visible copper, Cu_a, has two sulfur ligands and two nitrogen (or oxygen) ligands (Chan et al., 1978; Scott, 1982). Powers et al. (1981) have also proposed that Cu_{a_3} has at least one cysteine sulfur ligand, which in the oxidized protein bridges Cu_{a_3} and Fe_{a_3}. A problem arises when it is considered that the only cysteine residues conserved in cytochrome *c* oxidase from all five species listed above are Cys-196 and Cys-200 of subunit II. These residues are unreactive toward iodoacetamide in the native protein, consistent with their role as copper ligands (Darley-Usmar et al., 1982). The present finding that Glu-198 is involved in cytochrome *c* binding strongly suggests that Cys-196 and/or Cys-200 are liganded to Cu_a rather than Cu_{a_3}. It is expected that Cu_{a_3} would be located further away from the cytochrome *c* binding site. Other possible copper ligands in subunit II are to be found among the conserved histidine residues 24, 161, and 204 (Figure 6). It is noted that the subunit II sequence including Cys-196, Cys-200, and His-204 could potentially form a tight loop similar to the one involving the copper binding residues Cys-84, His-87, and Met-92 found in plastocyanin (Coleman et al., 1978). Met-206, which had originally been suggested as a possible copper ligand (Steffens & Buse, 1979), is conserved in the sequence of subunit II from four species but is replaced by threonine in maize.

Most workers have considered cytochrome *a* to be the initial electron acceptor in the reaction between ferrocytochrome *c* and cytochrome *c* oxidase (Wilson et al., 1975). However, Antalis & Palmer (1982) have shown in a recent study that cytochrome *a* and Cu_a are reduced with identical rate constants under a wide range of conditions. This could mean either that both accept electrons directly from cytochrome *c* or that the initial acceptor exchanges electrons very rapidly with the other component. The location of cytochrome *a* is not as well-defined as that of Cu_a but may also be located in subunit II or the interface between subunits I and II. The highly conserved sequence 104–115 is very unusual in that it contains alternating aromatic and acidic residues (Figure 6). These aromatic residues could potentially serve as a pathway for electron transfer from cytochrome *c*, known to bind at Asp-112 and Glu-114, to cytochrome *a*.

Poulos & Kraut (1980) have proposed that electron transfer from cytochrome *c* to cytochrome *c* peroxidase is facilitated by a system of parallel aromatic groups that form an overlapping π-orbital bridge between the two hemes. It is of interest that EDC can also modify carboxyl groups on cytochrome *c* peroxidase and inhibit the reaction with cytochrome *c* (Waldmeyer et al., 1982). Although the specific carboxyl groups modified have not yet been identified, the four adjacent acidic residues (32–35) that are located at the proposed cytochrome *c* binding site are obvious candidates.

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Waldmeyer et al. (1982) before publication.

Registry No. ETC, 22572-40-3; cytochrome *c* oxidase, 9001-16-5; cytochrome *c*, 9007-43-6; Asp, 56-84-8; Glu, 56-86-0.

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