# Different reactivity of carboxylic groups of cytochrome *c* oxidase polypeptides from pig liver and heart

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Received 5 June 1984; revised version received 19 June 1984

Cytochrome *c* oxidase isolated from pig liver and heart was incubated with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide and [$^{14}$C]glycine ethyl ester in the presence and absence of cytochrome *c*. Labelling of individual subunits was determined after separation of the enzyme complexes into 13 polypeptides by SDS-gel electrophoresis. Polypeptide II and additional but different polypeptides were labelled in the liver and in the heart enzyme. Labelling of polypeptide II and of some other polypeptides could be partially or completely suppressed by cytochrome *c*. From the data two conclusions can be drawn: In addition to polypeptide II, other polypeptides take part in the binding of cytochrome *c* to cytochrome *c* oxidase; the binding domain for cytochrome *c* is different in pig liver and heart cytochrome *c* oxidase.

*Cytochrome c oxidase isozyme* *Cytochrome c binding domain*
*1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide* *Tissue specificity*

## 1. INTRODUCTION

The mammalian cytochrome *c* oxidase complex is composed of 13 polypeptides [1–3]. Subunits I–III are of mitochondrial origin, whereas subunits IV–VIII are encoded in the nucleus and synthesized in the cytoplasm [3–6]. The mitochondrially synthesized polypeptides are suggested to represent the catalytic subunits. Cytochrome $a_3$ could be located in polypeptide I, cytochrome *a*, the binding site for cytochrome *c* and the two copper ions have been associated with polypeptide II, and the proton channel is probably contained in polypeptide III (review [3,6,7]). Since the specific function of the 10 cytoplasmically synthesized polypeptides is unknown, a regulatory role has been suggested for these polypeptides [3,4]. This suggestion was supported by the demonstration of different isozymes of cytochrome *c* oxidase occurring in various tissues of the same organism [2,3,8]. Different though similar N-terminal amino acid sequences were detected between 5 corresponding nuclear coded polypeptides of the enzyme from pig liver and heart [2], indicating the occurrence of multiple, tissue-specific genes for some polypeptides. In addition the kinetic properties, e.g., $V_{max}$ and $K_m$ for the high-affinity and low-affinity binding of cytochrome *c*, were found to be different for the bovine liver and heart cytochrome *c* oxidase [9]. We therefore suggest that some of the nuclear coded polypeptides might be involved in the binding of cytochrome *c* to the cytochrome *c* oxidase complex.

The protein-modifying reagent EDC, which reacts preferentially with carboxylic groups [10], has been used to cross-link subunit II with cytochrome *c* [11,12] and to label cytochrome *c* oxidase polypeptides by adding a radioactive nucleophile [13,14]. Addition of equimolar amounts of cytochrome *c* protected subunit II and some other polypeptides from labelling by

*Abbreviations:* EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; GEE, glycine ethyl ester; TMPD, *N,N,N',N'*-tetramethylphenylenediamine dihydrochloride



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[$^{14}$C]methyl-EDC [12] or [$^{14}$C]GEE in the presence of EDC [13]. It was concluded from this result that in addition to polypeptide II at least two other lower $M_r$ polypeptides contribute to the cytochrome *c* binding domain [13].

Here, the polypeptides located at the binding domain of cytochrome *c* are tentatively identified. It is demonstrated that this binding domain is different in the cytochrome *c* oxidase complex from pig liver and heart.

## 2. MATERIALS AND METHODS

Lauryl maltoside (lauryl $\beta$-D-maltopyranoside) was purchased from Calbiochem-Behring, Frankfurt, EDC, L-$\alpha$-phosphatidylcholine (type IVS, from soy bean) and horse heart cytochrome *c*, type VI from Sigma, München, and [$^{14}$C]GEE hydrochloride (50–60 mCi/mmol) from New England Nuclear, Dreieich.

Cytochrome *c* oxidase was isolated from pig liver and heart mitochondria as in [15] omitting the gel filtration step. The ammonium sulfate precipitate was dissolved in a small volume of 10 mM sodium phosphate (pH 7.0), 10 mM lauryl maltoside and dialyzed overnight against 10 mM sodium phosphate (pH 7.0). The clear dark green solution was stored frozen at −76°C.

Modification of cytochrome *c* oxidase with EDC was performed at 0°C for the indicated times in 10 mM sodium phosphate (pH 7.0), 5 mM lauryl maltoside, 2 mM EDC, 0.5 mM [$^{14}$C]GEE, 9 $\mu$M cytochrome *c* oxidase and the indicated amounts of cytochrome *c*. The reaction was stopped by addition of 1 M ammonium acetate (final conc. 0.1 M), and the enzyme was precipitated with acetone (80% final concentration). The precipitate was dissolved in sample buffer containing 8% SDS and 1% mercaptoethanol, and SDS gel electrophoresis was performed as in [1]. After staining with Coomassie blue R250 and destaining, the gel was incubated for 30 min in Enlightning (New England Nuclear) containing 5% glycerol, dried under vacuum and finally subjected to fluorography with XAR-5 Kodak film. The indicated bands of the dried gel were cut out, dissolved in 1 ml of 30% $H_2O_2$ containing 0.03 ml of 25% $NH_3$ for 24 h at 50°C, mixed with 10 ml Rotiszint 22 (Roth, Karlsruhe) and counted in a Berthold scintillation counter.

Cytochrome *c* oxidase activity was measured polarographically as in [16] in the presence of 25 mM Tris–acetate (pH 7.6), 7 mM Tris–ascorbate, 0.014 mM EDTA, 0.7 mM TMPD, 0.02–40 $\mu$M cytochrome *c* and 0.03 $\mu$M cytochrome *c* oxidase. The heme $aa_3$ content was determined from difference spectra (dithionite-reduced minus air-oxidized) using $\Delta\epsilon$(605–630 nm) = 24.0 $mM^{-1} \cdot cm^{-1}$ [17]. Heme *a*/protein ratios of the isolated enzymes were based on protein determination as in [18].

## 3. RESULTS

The kinetic properties of the isolated cytochrome *c* oxidases from pig heart and liver were measured polarographically at various cytochrome *c* concentrations in the presence of TMPD as described in section 2. The $K_m$ and $V_{max}$ values were determined graphically from Eadie-Hofstee plots according to [16]. The two enzymes had very similar kinetic behavior. The $K_m$ for the high-affinity binding of cytochrome *c* was 52 and 55 nM; the $K_m$ for the low-affinity binding of cytochrome *c* was 1.5 $\mu$M for both, and the $V_{max}$ 170 and 180 $s^{-1}$ (mol cytochrome *c*/mol cytochrome $aa_3$) for the heart and liver enzyme, respectively. The heme *a*/protein ratios of the enzymes (nmol/mg protein) were 9.5 for the heart and 8.7 for the liver. The visible spectra of the two enzymes were indistinguishable.

The water-soluble carbodiimide EDC was used to investigate the binding domain of cytochrome *c* in the two enzyme complexes. After activation by EDC, accessible carboxylic groups can be labelled with [$^{14}$C]GEE by formation of a peptide bond with the ester. Cytochrome *c* was found to protect polypeptide II and some additional polypeptides from becoming labelled [12,13]. To determine whether protection by cytochrome *c* was nonspecific, pig liver cytochrome *c* oxidase was labelled with EDC and [$^{14}$C]GEE in the presence of increasing cytochrome *c* concentrations as shown in fig.1. In the absence of cytochrome *c* most polypeptides are labelled. With increasing cytochrome *c* concentration a reduction of labelling is found for polypeptides II, VIa-c, VIIa and VIII. This protective effect of cytochrome *c* is complete at a molar cytochrome *c*:cytochrome *c* oxidase ratio of 1:1 and occurs in parallel for all

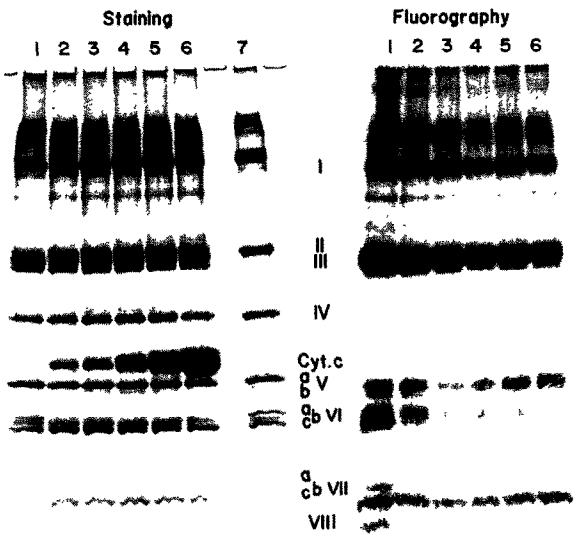


Fig.1. Influence of cytochrome *c* on the labelling of pig liver cytochrome *c* oxidase polypeptides by EDC and [$^{14}$C]GEE. Pig liver cytochrome *c* oxidase was incubated for 20 h at 0°C with increasing concentrations of cytochrome *c*. Conditions for further sample treatment, SDS gel electrophoresis, staining, and fluorography are given in section 2. Lanes 1–6: samples incubated at a molar cytochrome *c*/cytochrome *c* oxidase ratio of 0, 0.6, 1, 2, 4 and 8, respectively. Lane 7: untreated enzyme.

protected polypeptides. At an 8-fold molar excess of cytochrome *c* no additional protection is observed, suggesting that the protection is due to a specific and stoichiometric binding of cytochrome *c*.

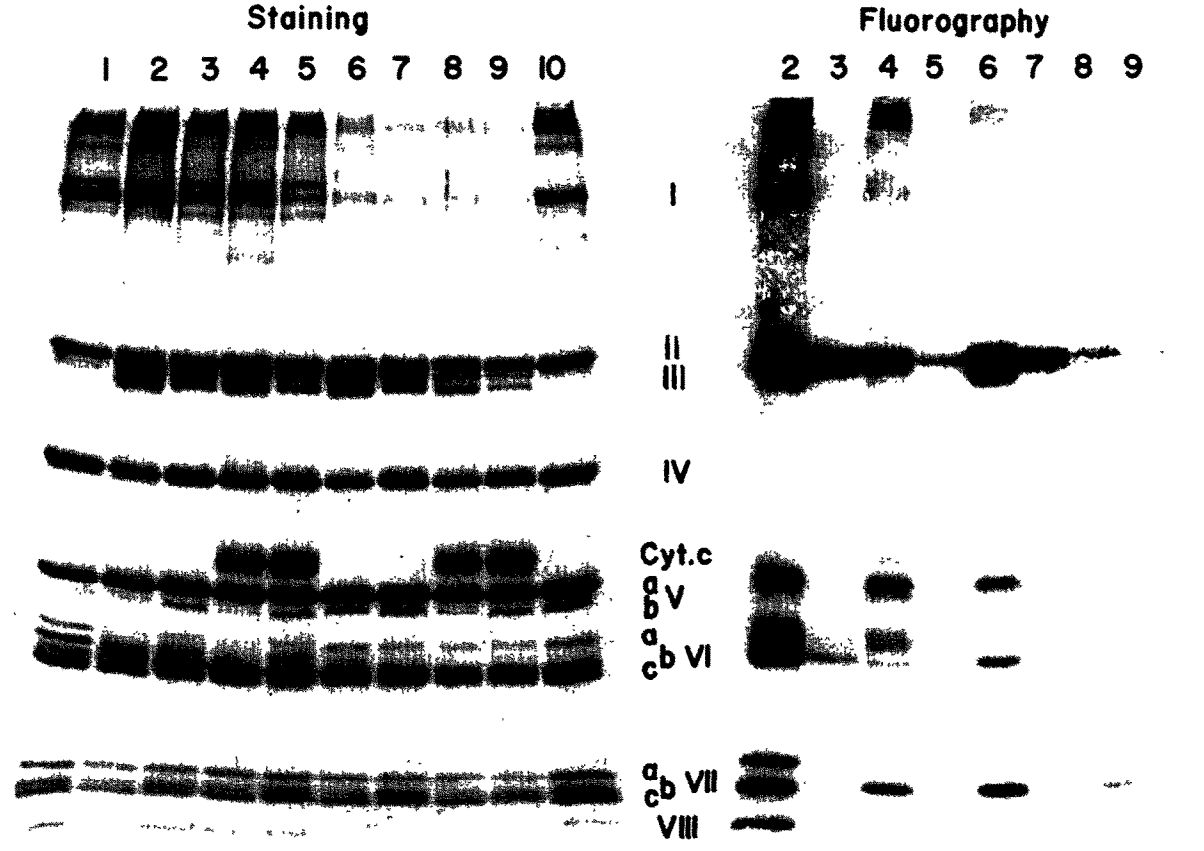


Fig.2. Comparison of labelling of pig liver and pig heart cytochrome *c* oxidase polypeptides with EDC and [$^{14}$C]GEE. Pig liver (lanes 1–5) and pig heart cytochrome *c* oxidase (lanes 6–10) were incubated for 1.5 h (lanes 3,5,7,9) and 20 h (lanes 2,4,6,8) in the absence (lanes 2,3,6,7) and presence (4,5,8,9) of cytochrome *c* (1:1 molar ratio) as described in section 2. Lanes 1,10: untreated enzymes. Conditions for SDS-gel electrophoresis, and fluorography are described in section 2.

Since the N-terminal amino acid sequences of polypeptides VIa, VIIa-c and VIII of pig liver and heart cytochrome *c* oxidase are different [2], it was of interest to compare the labelling pattern of the two enzymes. In fig.2 the Coomassie blue staining pattern of the isolated enzymes is shown before and after incubation with EDC and [$^{14}$C]GEE for 1.5 and 20 h in the presence and absence of cytochrome *c*; the labelling pattern is shown for comparison. The untreated liver enzyme differs from the heart enzyme in that polypeptide VIa is split into two bands and polypeptide VIII is not in the same position. The additional band in the region of polypeptide I we believe only to be an impurity. The labelling pattern of the heart enzyme is clearly different from that of the liver enzyme. Polypeptides VIa, VIIa and VIII, which are strongly labelled in the liver enzyme, are only labelled to a small extent in heart cytochrome *c* oxidase in the presence or absence of cytochrome *c*.

It should be noted that chemical modification leads to a slight change of mobility of several polypeptides. Polypeptide II is split into 3 new bands. The position of the radiolabelled bands of some polypeptides appears slightly above the Coomassie blue-stained bands due to binding of the nucleophile (fig.2). This was verified by careful comparison of the location of stained bands on several dry gels with the radioactive bands on the corresponding films.

To quantitate the labelling and protection by cytochrome *c*, the bands were cut out and counted. When the incubation with EDC and [$^{14}$C]GEE was carried out for 20 h, it was found that the radioactivity of all bands was increased by a factor of approx. 2.5 over the values at 1.5 h (not shown). The data of fig.2 from samples incubated for 20 h are presented in fig.3 together with data obtained under identical conditions except that the incubation was performed in the presence of 60 mM lauryl maltoside or 60 mM lauryl maltoside plus 0.75% L-$\alpha$-phosphatidylcholine. The nonionic detergent caused a drastic reduction in the labelling of polypeptide II in the absence of cytochrome *c*. Since the labelling in the presence of cytochrome *c* was relatively greater for polypeptide II and most other polypeptides, there was virtually no protection by cytochrome *c* in either enzyme. These detergent effects are completely reversed by inclusion of the phospholipid during the incubation.

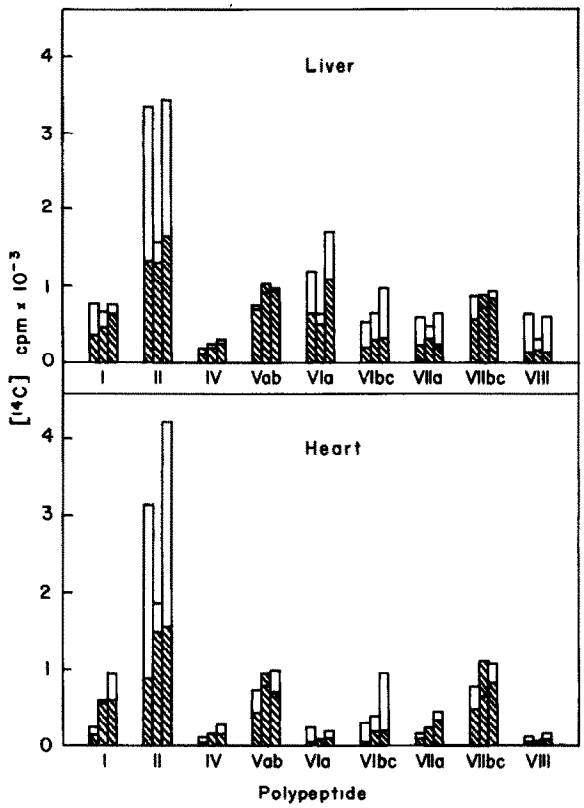


Fig.3. Effect of phospholipid and nonionic detergent on the labelling by EDC and [$^{14}$C]GEE of cytochrome *c* oxidase polypeptides in the presence and absence of cytochrome *c*. Pig liver and heart cytochrome *c* oxidase were incubated for 20 h with EDC and [$^{14}$C]GEE and SDS-gel electrophoresis, fluorography and identification and counting of labelled bands were done as described in section 2. The 3 bars for each polypeptide represent the labelling obtained under 3 different conditions: left bar (data from fig.2), control; middle bar, addition of 60 mM lauryl maltoside; right bar, addition of 60 mM lauryl maltoside and 0.75% L-$\alpha$-phosphatidylcholine. The hatched bars represent the labelling in the presence of cytochrome *c* (1:1 molar ratio), the open bars in its absence.

For example, with phospholipid present, the labelling of polypeptide VIbc is increased above the level of labelling in the absence of detergent and phospholipid. Since the labelling in the presence of cytochrome *c* is unaffected, there is a relatively greater protection. Phospholipids are known to be required for full enzymatic activity of isolated cytochrome *c* oxidase [14]. Therefore, it may be concluded that the protection by cytochrome *c* of

labelling of polypeptides II, VIa, VIbc, VIIa and VIII by EDC and [$^{14}$C]GEE reflects the native conformation of the polypeptides within the enzyme complex, which is different in the liver and heart enzyme.

In further studies the effect of various parameters on the labelling patterns of the liver and heart enzymes were investigated. The labelling patterns were found to be rather reproducible. In the pH range 6.15–7.65 optimum cytochrome *c* protected labelling was found at pH 7.0 for all polypeptides (not shown).

Various nucleotides at 5 mM and phosphate did not change the labelling pattern (not shown). Only at above 50 mM did phosphate cause a reduction of labelling for all polypeptides (not shown).

Finally, the stoichiometry of labelling was studied by measuring the incorporation of GEE as a function of its concentration. It was shown in [14] that at least 3 different carboxylic groups of polypeptide II from beef heart cytochrome *c* oxidase are modified by 1-ethyl-3-[3-(trimethylamino)propyl]carbodiimide. As shown in part in fig.4, at all concentrations of GEE, substoichiometric incorporation and no saturation for all polypeptides are found. At high GEE concentrations a reduction of the percentage of cytochrome *c* protected reaction of the nucleophile is found, which may be explained by its competition with cytochrome *c* (5000-fold excess of GEE).

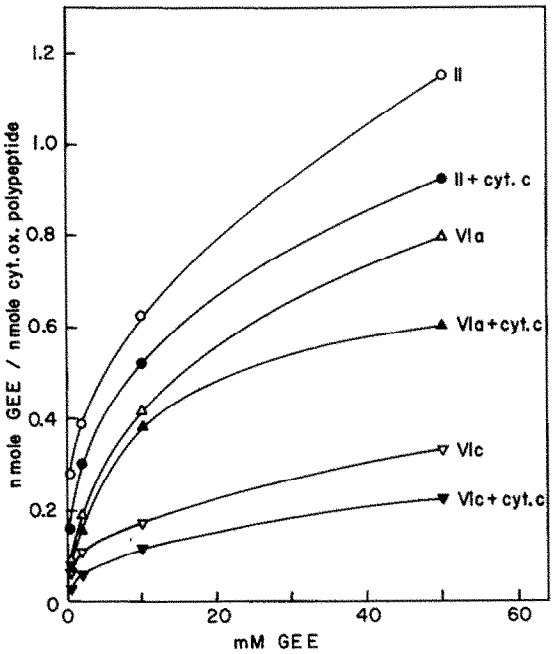


Fig.4. Reaction of GEE and EDC with different polypeptides of pig liver cytochrome *c* oxidase in the presence and absence of cytochrome *c* at increasing concentrations of the nucleophile. Incubation of the enzyme was performed in the presence and absence of cytochrome *c* (1:1 molar ratio) with 0.5 mM [$^{14}$C]GEE for 20 h as described in section 2. Where indicated, [$^{14}$C]GEE was mixed with unlabelled nucleophile to the final concentrations of 2, 10 and 50 mM GEE. SDS gel electrophoresis, identification and counting of labelled bands as described in section 2.

## 4. DISCUSSION

The binding domain of cytochrome *c* at the cytochrome *c* oxidase complex has been investigated by using cross-linking reagents [20–25], carboxylic group modifying reagents [11–13] and monosubstituted lysine derivatives of horse cytochrome *c* [26–32]. Polypeptides II [12,14,22, 23], III [20,21,25], IV [11], and a polypeptide with an apparent $M_r$ of 15000 [24] have been suggested to be involved in the binding of cytochrome *c*. Authors in [13] concluded from their labelling pattern of cytochrome *c* oxidase polypeptides (obtained with EDC and [$^{14}$C]GEE in the presence and absence of cytochrome *c*) that in addition to polypeptide II at least two other low-$M_r$ polypeptides contribute to the cytochrome *c* binding domain.

The lysine residues of cytochrome *c* involved in the binding to bovine heart cytochrome *c* oxidase have been identified either by use of cytochrome *c* derivatives [26–31] or by differential chemical modification [32]. Five of the 19 lysine amino groups of cytochrome *c* were found to contribute strongly, 7 slightly, and the remaining 7 not at all to the electrostatic interaction with cytochrome *c* oxidase [26,29,30]. Reaction of bovine heart cytochrome *c* oxidase with 1-ethyl-3-[2-[$^{14}$C](trimethylamino)propyl]carbodiimide leads to labelling of 3 carboxylic groups of polypeptide II (Asp-112, Glu-114, Glu-198); cytochrome *c* protects against almost all of this labelling. The labelling was accompanied by inhibition of activity [14]. In that study maximally 7 carboxylic groups of polypeptide II were considered as possible participants in cytochrome *c* binding. Thus, it appears

that in addition to carboxylic groups of polypeptide II those from other polypeptides might also be involved in the binding of cytochrome *c*.

Here, an SDS-gel electrophoretic system capable of separating all 13 polypeptides of mammalian cytochrome *c* oxidase [1] was used; the polypeptides labelled by EDC and [$^{14}$C]GEE were then identified on these gels. Protection of labelling by cytochrome *c* was found for polypeptide II and for several other polypeptides with stoichiometric amounts of cytochrome *c*. An 8-fold excess of cytochrome *c* did not further suppress the cytochrome *c*-insensitive labelling, thus excluding an unspecific protection of labelling by cytochrome *c*. Although the visible spectra, heme *a*/protein ratios and kinetic properties of the two enzymes (measured polarographically in the presence of TMPD) were essentially identical, the cytochrome *c* protected labelling pattern of the polypeptides was clearly different. Three polypeptides (VIa, VIIa, VIII) are protected by cytochrome *c* mainly in the liver enzyme. Polypeptide VIc exhibits cytochrome *c*-sensitive labelling in both enzymes, whereas labelling of polypeptide Vab (mainly Vb) was protected by cytochrome *c* only in heart. Polypeptides IV, Va, VIb and VIIc were only weakly labelled in both enzymes. The above conclusions are based on careful examination of the labelled bands of a large number of gels.

The different reactivity of liver and heart cytochrome *c* oxidase polypeptides, as described above, is consistent with the existence of different N-terminal amino acid sequences in the corresponding nuclear coded polypeptides of bovine liver and heart cytochrome *c* oxidase [2,3,8]. Although the specific function of the various polypeptides remains to be explained, the above described results further establish the occurrence of tissue-specific isozymes of cytochrome *c* oxidase in higher organisms.

## ACKNOWLEDGEMENT

This investigation was supported by the Deutsche Forschungsgemeinschaft (SFB 103, A2).

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