STOICHIOMETRIC LABELING AND INHIBITION OF CYTOCHROME *c* OXIDASE BY PHENYLISOTHIOCYANATE

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1. Introduction

Cytochrome *c* oxidase, the terminal component of the respiratory chain, is a protein complex associated with the inner mitochondrial membrane. Labeling studies have indicated that it consists of multiple subunits, some of which are externally associated with the membrane while others are deeply embedded into the lipid bilayer [1]. Most of the labeling reagents used to date have, however, either broad specificity or, as in the case of azido derivatives, no specificity [2]. Studies of the relevance of specific reactive groups for enzymatic function become therefore difficult. Phenylisothiocyanate (PITC) has been described as a group specific hydrophobic reagent [3]. Chemical groups of specific reactivity are highly relevant for the functioning of membrane protein, an example being the unique carboxyl group of the ATPase proteolipid covalently labeled by dicyclohexyl-carbodiimide [4]. PITC was employed in the hope of identifying a functionally important and unique reactive group in cytochrome *c* oxidase. This study will describe the covalent labeling of cytochrome *c* oxidase by PITC with concomitant inhibition of its enzymatic activity.

2. Methods and materials

Cytochrome *c* oxidase was prepared as in [5] and had a heme content of 10 nmol/mg protein. Oxygen consumption was determined polarographically using a standard Clark electrode equipped with a thermostated chamber [5]. The PITC-labeling procedure and conditions are given under the appropriate tables and figure. In all experiments the enzyme was suspended in a 50 mM sodium phosphate buffer, pH 7.0. Protein was determined by the Lowry method [6] in the presence of 0.1% SDS. Polyacrylamide–dodecyl sulfate disc electrophoresis was performed as in [7] utilizing 10% and 15% crosslinked gels. When PITC-labeled samples were electrophoresed, identical samples were applied to separate gels. One gel was stained, the duplicate gel was immediately cut into 1 mm slices. Individual gel slices were extracted overnight with 10 mM Triton-X-100 and then counted.

[^{14}C]-PITC was purchased from Amersham Radiochemicals and had spec. act. 11 mCi/mmol. All other chemicals and reagents used were of the highest purity commercially available.

3. Results and discussion

3.1. *Effect of temperature on the inhibition of cytochrome* c *oxidase activity by PITC*

As shown in table 1, PITC effectively inhibited cytochrome *c* oxidase activity over 10–25°C. Preincubation with the inhibitor at 4°C yielded no inhibition while preincubation at 37°C greatly reduced enzymatic activity in both the control and PITC-treated samples. Incubation of the enzyme under these conditions resulted in extensive aggregation, however. Inhibition of enzymatic activity by PITC was found to be maximal at 60 min of preincubation and approximately half-maximal after 30 min of preincubation with the inhibitor (data not shown).

Table 1
Inhibition of cytochrome *c* oxidase by PITC

Temp. (°C)	Control (nequiv. e^- s^{-1}) nmol $heme^{-1}$	PITC	% Inhibition
4	51	51	0
10	67	7	90
15	68	9	87
20	73	8	89
25	73	7	90
37	19[a]	20[a]	0

[a] Extensive aggregation of enzyme was noted

PITC (635 nmol) was added to cytochrome *c* oxidase (1 mg) and incubated at the given temperatures for 60 min. Enzymatic activity was determined polarographically at 20°C

3.2. *Incorporation of PITC into cytochrome* c *oxidase*

A mol/mol ratio of 1 for covalent incorporation of PITC into oxidized cytochrome oxidase occurred when the preincubation was carried out from 10–25°C (table 2). Very low incorporation was noted at 4°C whereas elevated labeling was observed at 37°C. The extensive aggregation of the enzyme noted under these conditions may result in shielding by protein–protein interactions of water accessible residues which then become susceptible to PITC labeling. Contrastingly, greatly reduced binding of PITC was observed when the enzyme was reduced by dithionite. Significant binding was noted only with the aggregated reduced enzyme at 37°C. In control experiments with test substrates, PITC labeling was not affected by the presence of sodium dithionite.

Table 2
Incorporation of PITC into cytochrome *c* oxidase

Temp. (°C)	Oxidized enzyme (mol PITC/mol cytochrome *c* oxidase)	Reduced enzyme
4	0.21	0.18
10	0.85	0.20
15	0.99	0.26
20	0.98	0.25
25	1.02	0.31
37	3.40	1.20

Labeling conditions were identical to those in table 1 with the exception that 1 mM sodium dithionite was used for reduction of cytochrome *c* oxidase. The degree of reduction was monitored spectrophotometrically. After a 60 min preincubation period with PITC aliquots of the enzyme were removed and immediately precipitated by addition of cold acetone. The precipitated enzyme was washed once with acetone and suspended in sodium dodecyl sulfate for protein and radioactivity measurements

3.3. *Location of PITC-binding within the cytochrome* c *oxidase complex*

Figure 1 shows a densitometric trace of the polypeptide profile of PITC-labeled cytochrome oxidase obtained by polyacrylamide–dodecyl sulfate gel electrophoresis. Covalent incorporation of the radio-

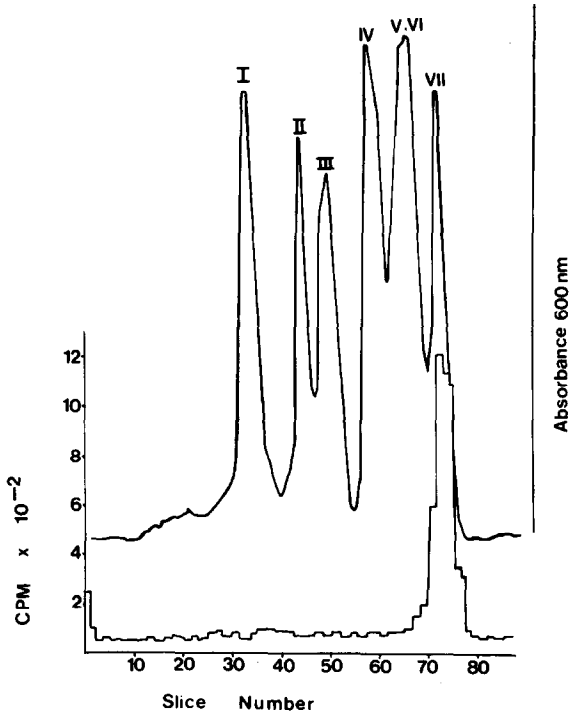

Fig.1. Electrophoretic analysis of PITC-labeled cytochrome *c* oxidase. Cytochrome *c* oxidase was labeled with [^{14}C]PITC (635 nmol/mg enzyme) for 60 min at 20°C. The enzyme was precipitated with a saturated ammonium sulfate solution, suspended in water and re-precipitated. The precipitate was suspended in SDS when the electrophoresis was immediately performed or stored at −70°C without the detergent. While the polypeptide profile shown here was obtained with 10% crosslinking, identical labeling results were obtained with 15% polyacrylamide gels. 50 μg protein were applied. Roman numerals are used to indicate the various polypeptides as in [8]. Radioactive content is displayed below the densitometric trace.

active label occurred only in one polypeptide, subunit VII, with app. mol. wt 8000. While binding of PITC was greatly diminished in the dithionite-reduced enzyme, the location of the binding was identical to that in the oxidized enzyme. Additionally, electrophoresis was carried out of acetone-precipitated PITC-labeled enzyme. Again, subunit VII was the only polypeptide labeled. However, because of aggregation between subunits I and III that occurred upon acetone precipitation, ammonium sulfate precipitation was preferred for electrophoretic analysis. It must be noted that ammonium sulfate precipitation did not remove all of the noncovalently bound label. If the enzyme was left for extended periods of time in detergent in the presence of non-reacted PITC all of the subunits of the enzyme eventually became labeled to varying degrees. In contrast, acetone precipitation appeared to remove all noncovalently bound label.

4. Conclusions

PITC appeared to inhibit cytochrome *c* oxidase activity only under conditions where covalent binding occurred. Preincubation of the enzyme with PITC at 4°C yielded only negligible binding and no inhibition of activity. Subjection of cytochrome oxidase to the hydrophobic label at higher temperatures resulted in inhibition of enzymatic activity and a 1:1 stoichiometry of inhibitor to enzyme. Preincubation of the enzyme at 37°C produced loss of enzymatic activity, aggregation of the enzyme and increased binding, most probably due to a change in the environment of some of the exposed residues. It is highly interesting that under conditions when a 1:1 molar ratio of bound inhibitor to enzyme was noted in the oxidized form, only 0.2 mol phenylisothiocyanate was bound per mol reduced cytochrome oxidase.

PITC may be considered highly hydrophobic in that it partitions 97% into hexane in a hexane:water mixture. In membrane labeling studies, maintenance of external pH 7 afforded protection of aqueous-accessible groups while labeling of only known transmembrane proteins occurred [3]. We propose, in an analogous fashion, that PITC may be binding to an amino group which is essential for functional activity and situated in an apolar environment on subunit VII. The specific labeling of cytochrome oxidase subunit VII by PITC with concomitant loss of enzyme activity suggests that a functionally important lysyl group may be located in this subunit. The decrease in the reactivity of the PITC-labeled groups observed upon reduction of the enzyme suggests vicinity of this group to one of the redox centers of the enzyme. Alternatively, a redox mediated conformational change of the enzyme may affect the environment of the PITC-labeled group making it more or less susceptible to chemical reactivity. It is interesting to compare these results with those in [9] which indicate the possibility that in one of the low molecular weight subunits of yeast cytochrome oxidase an ϵ-amino group may form a Schiff base with the formyl group of heme *a*. Work is in progress to establish whether the group labeled by PITC is close to heme *a*, and thus functionally important for the activity of the enzyme.

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