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ON THE IDENTIFICATION AND NOMENCLATURE OF THE POLYPEPTIDE SUBUNITS OF BOVINE CYTOCHROME C OXIDASE

Victor M. Darley-Usmar* and Michael T. Wilson†

Institute of Molecular Biology, University of Oregon, Eugene, OR 97403 U.S.A.

†Dept. of Chemistry, University of Essex,
Wivenhoe Park, Colchester CO4 3SQ U.K.

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SUMMARY: The polypeptide subunits of cytochrome c oxidase (E.C. 1.9.3.1.) isolated from beef heart which react with ^{203}[Hg]Cl$_2$ have been identified. A rapid and simple method for identifying and numbering the subunits is described which is independent of the type of sodium dodecyl sulphate-polyacrylamide gel system employed to separate them.

Cytochrome c oxidase (E.C. 1.9.3.1.), a major protein component of the inner mitochondrial membrane and the terminal electron acceptor of the respiratory chain, is a complex enzyme consisting of several polypeptides (1,2). The number of polypeptides has been determined by SDS[1] gel electrophoretic techniques and their nomenclature is based upon their order of migration in these gel systems (2,3,4,5). As the order of migration is dependent on the conditions used, this method of identifying individual polypeptides naturally leads to some confusion (3,6,7).

In this paper we compare the polypeptide numbering systems of various research groups and present a scheme for unequivocally identifying a given polypeptide. Assignment rests on three simple criteria determined from SDS electrophoresis: a) gross apparent molecular weight; b) sensitivity to Trypsin treatment; c) mercury binding properties. This latter criterion involves reaction of the oxidase with ^{203}[Hg] followed by autoradiography of the resulting SDS gels (8).

[1]Abbreviations used in this paper are following: SDS, sodium dodecyl sulphate, PGGE, polyacrylamide gradient gel electrophoresis.

*Author to whom correspondence should be directed.

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METHODS: Cytochrome c oxidase was prepared from beef heart mitochondria by the method of Yonetani (9).

Preparation of protease treated cytochrome oxidase. Trypsin treated oxidase was prepared by incubating 0.5 ml of 280 μM oxidase (total haem) in 0.1 M Na phos pH 7.4, 1% Tween 80 with 0.5 mg of trypsin for thirty minutes at room temperature. Trypsin and proteolytic fragments were separated from oxidase on a Sephadex G.75 column (1 x 30 cm) equilibrated with 0.1 M sodium phosphate 1% Tween 80 pH 7.4. Control samples of oxidase were treated identically but without trypsin.

Reaction with mercuric chloride and SDS-PGGE*. Samples of oxidase after gel filtration were treated with mercuric chloride (^{203}[Hg]Cl_2) by incubation at room temperature for thirty minutes. In a typical experiment 100 μl of cytochrome c oxidase (40 μM total haem) was mixed with 5-40 μl of ^{203}[Hg]Cl_2 (1.2 mM in 1N HCl, 0.18 Ci/M). After incubation 25 μl of oxidase samples (∿ 100 μg of protein) were mixed with denaturing solutions containing 5% SDS, 0.25 M Tris/HCl pH 6.2, 8 M urea with or without 2-mercaptoethanol (57 mM). SDS-PGGE and autoradiography was conducted as described previously (2,8). Autoradiograms and gels were scanned at 600 nm using a Perkin-Elmer 575 spectrophotometer.

RESULTS:

The effect of proteolysis on the polypeptide banding pattern. Figure 1a shows the polypeptide banding pattern of bovine cytochrome c oxidase as prepared and Fig. 1b after trypsin treatment. Differences are evident only among the low molecular weight polypeptides. Digestion has removed only two polypeptides which is in reasonable agreement with the results of Ludwig et al. (6). Subunit III stains less intensely with coomassie blue than the other polypeptides and migrates as a broad band on SDS gel electrophoresis (Fig. 1a). Both these observations have been noted previously by Downer et al. (3).

Labelling of the subunits of cytochrome c oxidase with mercuric chloride. In these experiments two conditions for labelling were used. Following incubation at room temperature with ^{203}[Hg]Cl_2 the enzyme sample was divided into two aliquots. Each aliquot was prepared for SDS gel electrophoresis by incubation in a denaturing medium in only one of which, however, 2 mercaptoethanol (57 mM) was present. These treatments result in different distributions of radioactive mercury among the subunits.

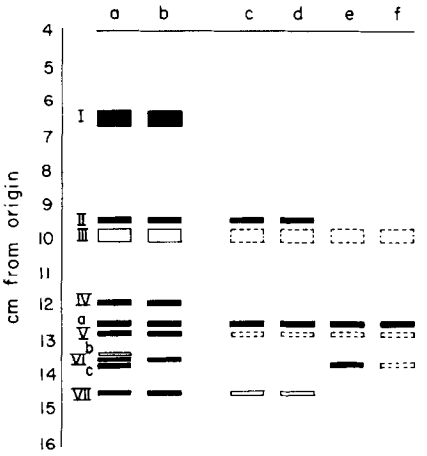

Fig. 1. Protein and ^{203}[Hg] Banding Patterns after SDS-Polyacrylamide Gradient Gel Electrophoresis.

Figs. a and b show the protein banding pattern of coomassie blue stained samples. Fig. a shows beef cytochrome c oxidase as prepared and Fig. b after proteolytic treatment. Figs. c-f show traces of autoradiograms of ^{203}[Hg] labelled oxidase (Hg:haem a = 4:1). Other conditions were as follows: Figs. c and d show autoradiographs of samples labelled in the absence of 2-mercaptoethanol and Figs. e and f in its presence. Figs. d and f show autoradiograms of trypsin treated samples. Three levels of coomassie blue staining or ^{203}[Hg] labelling are shown in Fig. 1. These are:

high labelling or stain

moderate labelling or stain

low labelling

Figs. 1c and 1d compare the ^{203}[Hg] labelling pattern of the control and the trypsin treated enzyme in the absence of mercaptoethanol. We have discussed this labelling pattern in a previous publication and shown that mercury reacts specifically with cysteine residues (8). Using the numbering system of Ludwig et al. (6) the three labelled polypeptides are subunit II, polypeptide a, and subunit(s) VII. The trypsin treated oxidase and the untreated enzyme show no significant differences in either the amount of label bound or its distribution.

In contrast the labelling pattern of the same protein samples when incubated with mercaptoethanol do show some differences. These are shown in Figs. 1c and 1e which compare labelling of the control enzyme under the two condi-

Table 1

Comparison of the polypeptide numbering systems of cytochrome oxidase

Wilson et al. (2)		Downer et al. (3)		Steffens & Buse (11)		Yu & Yu (4)		Number of Cysteine residues	
No.	Mr	No.	Mr	No.	Mr	No.	Mr		(ref)
I	35,700	I	35,400	I	36,000	I	40,000		
II	22,500	II	24,100	II	24,000	II	21,000	2	(11)
III	21,100	III	21,000	III	19,000			2	(16)
IV	16,900	IV	16,800	IV	16,000	III	14,800	0	(12)
a	12,500	a	nr	V	11,600	IV	13,500	3	(17)
V	11,700	V	12,400	VI	12,500	V	11,600	1	(13)
b	10,500	b	nr		nr				
VI	7,600	VI	8,200		nr	VI	9,500		
c	7,000	c	nr	VII	9,500			4	(14)
VII	4,500	VII	4,400	VIII	6,000	VII	7,600		

The polypeptides are arranged so that the same polypeptide, whatever its designated number is in the same horizontal row.

nr - not reported

tions. The label on subunit II and VII has been displaced (Fig. 1e) and a low molecular weight polypeptide has become labelled.

Figure 1f shows the protease treated sample which also shows a mobilisation on the addition of mercaptoethanol (Cf 1d). The newly labelled lower molecular weight polypeptide of the control sample (1e) is substantially removed by trypsin and so the extent of labelling is correspondingly less.

DISCUSSION: It is now generally accepted that the full functional activity of bovine oxidase is associated with 6-10 polypeptides (2,5,6,10). The polypeptides have been identified and numbered by the convention of labelling them in order of decreasing molecular weight. Examples from a number of workers are shown in Table 1. Unfortunately the behaviour of the polypeptides of cytochrome oxidase is not the same in all the gel systems currently in use. Changes in the order of migration and resolution have been noted for subunits V and VI and subunits II and III (3,7). In addition some workers, notably Ludwig et al. (6) have designated some polypeptides as contaminants

since they can be removed by limited proteolysis without change in the activity of the enzyme (6).

It has become increasingly important for different groups to correlate their experimental results. To do this on the basis of molecular weight alone is not satisfactory particularly for those polypeptides with molecular weight below 16000 Daltons.

Here we have presented data by which each polypeptide can be discriminated on the basis of its apparent molecular weight, resistance or sensitivity to proteolysis and labelling with radioactive mercury.

We have assigned our banding pattern found on SDS-PGGE in the following way: Subunit I, II and III are discriminated by the high molecular weight of subunit I ($\sim$ 35,000 Daltons), the reaction of subunit II with mercury at low concentrations of mercury (relative to haem a e.g., 1 Hg atom/haem group) and its slightly higher apparent molecular weight than subunit III (Table 1) which binds mercury only at high concentration (8). Subunit II has two cysteine residues (positions 196 and 200) one of which is a putative copper ligand (11). We conclude therefore that as this subunit is clearly preferentially labelled by the mercury the copper site must be very near the surface of the molecule (8). Subunit IV does not bind mercury [no cysteines are present in the sequence (12)], and stains strongly with coomassie blue. The next polypeptide has been numbered as 'a' (6), IV (4) and V (5) by various groups (see Table 1). This polypeptide reacts strongly with mercury suggesting a large number of free thiol groups. For this reason we can discriminate it from subunit V which from the sequence has only one cysteine residue (13). Subunit V is labelled to a small extent with mercury but only at high $^{203}[Hg]Cl_2$ concentrations. Of the next three polypeptides two are removed by limited proteolysis and have been named contaminants b and c by Ludwig et al. (6). Recently we have learned (Buse - personal communication) that polypeptide c corresponds to the polypeptide numbered VII by Buse, which has four cysteine residues (14). In view of

the fact that this polypeptide is labelled quite strongly in the presence of the disulphide reducing reagent mercaptoethanol it is possible that the cysteines form disulphide bonds (14). Subunit VI is neither removed by trypsin treatment nor labelled by ^{203}[Hg]. The last low molecular weight fraction, subunit VII, is an heterogeneous component consisting of three polypeptides (15). The two sequenced polypeptides (N-terminal amino acids Ileu and Ser) of these three shows no cysteine residues but ^{203}[Hg]-labelling does occur which indicates that the other polypeptide contains cysteine residues (G. Steffens personal communication).

Table 1 compares the numbering, molecular weight and, where known, the number of cysteine residues of the polypeptides of bovine cytochrome oxidase. It is clear from Table 1 that laboratories working on oxidase have adopted different labelling systems such that a given polypeptide may be referred to in the literature by more than one number. The unambiguous way to identify subunits is through their amino acid sequence or, failing that, through N-terminal analysis. The former technique is not routine in most laboratories and the latter method is made difficult with cytochrome oxidase as many of the polypeptides have blocked N-terminal residues (5). Here we offer a simple and readily available method for identifying subunits and a comparison between the nomenclature of different groups so that a variety of different gel systems may be employed.

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