IDENTIFICATION OF OXIDATION FACTOR AS A RECONSTITUTIVELY ACTIVE FORM OF THE IRON–SULFUR PROTEIN OF THE CYTOCHROME b–c_1 SEGMENT OF THE RESPIRATORY CHAIN

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1. Introduction

Oxidation factor is a protein which is required for electron transfer in the succinate–cytochrome c reductase segment of the mitochondrial respiratory chain. The activity of this protein was discovered during attempts to reconstitute the succinate–cytochrome c reductase segment from resolved components [1,2]. From work with the partially purified protein, oxidation factor is known to be required for succinate–cytochrome c reductase and ubiquinol–cytochrome c reductase activities [1–3] and for reduction of cytochrome c_1 by succinate [4] and ubiquinol [2]. However, further details of how oxidation factor participates in respiration have remained obscure, since the partially purified protein was not shown to contain any prosthetic group capable of oxidation–reduction [2].

The purpose of this paper is to report that oxidation factor is a reconstitutively active form of the iron–sulfur protein of the cytochrome b–c_1 segment first discovered [5] and now shown to be required for electron transfer from succinate or ubiquinol to cytochrome c. This is the first time this iron–sulfur protein has been purified in a biologically active form, and the reconstitution of electron transfer activities provides unequivocal evidence that this iron–sulfur protein is an obligatory component of the respiratory chain.

2. Materials and methods

Succinate–cytochrome c reductase [1], ubiquinol–cytochrome c reductase [6], succinate dehydrogenase [7], and succinate–ubiquinone reductase [7] activities were measured at 30°C. One unit is defined as 1 μequiv./min. Oxidation factor activity was measured by a reconstitution assay in which it restores cytochrome c reductase activity to depleted reductase complex [2] as shown below. Succinate–cytochrome c reductase complex was depleted of oxidation factor as in [1,2], except that the amount of guanidine used for the extraction was 1.30 M and hydrosulfite, rather than succinate, was included as a protective agent. The depleted complex has both cytochromes b and c_1 in the same ratio as the parent complex (see fig.1 in [4]). In the presence of phospholipid, ubiquinone-10, and succinate dehydrogenase the depleted complex has succinate–cytochrome c reductase activity of only 0.1–0.2 units/mg, and this is restored to 5–6 units/mg in saturable fashion by reconstitution with oxidation factor as shown below.

Although the method for purification of reconstitutively active iron–sulfur protein (oxidation factor) will be the subject of a forthcoming paper (B.L.T., C.A.E., in preparation) a brief summary of the procedure is relevant to the current report. Oxidation factor was extracted from reductase complex [1] with 1.50 M guanidine in the presence of 0.7% cholate and dialyzed to remove cytochrome c_1 [2]. The soluble protein was then fractionated with ammonium sulfate and recovered as a 25–40% ammonium sulfate pellet, referred to herein as 25-40p. The 25-40p fraction was then chromatographed on hydroxyapatite and purified, reconstitutively active iron–sulfur protein was eluted by 100–125 mM

phosphate (pH 7.0) from a 0–200 mM linear gradient.

Nonheme iron [8], acid-labile sulfide [9] and protein [10] were measured by standard procedures and acrylamide gel electrophoresis in dodecyl sulfate as in [11].

3. Results and discussion

Oxidation factor was discovered as a protein which restores succinate–cytochrome *c* reductase activity to reductase complex from which it is extracted by guanidine and cholate [2]. This reconstitutive activity was used as an assay to follow the purification of oxidation factor as illustrated in fig.1, which shows

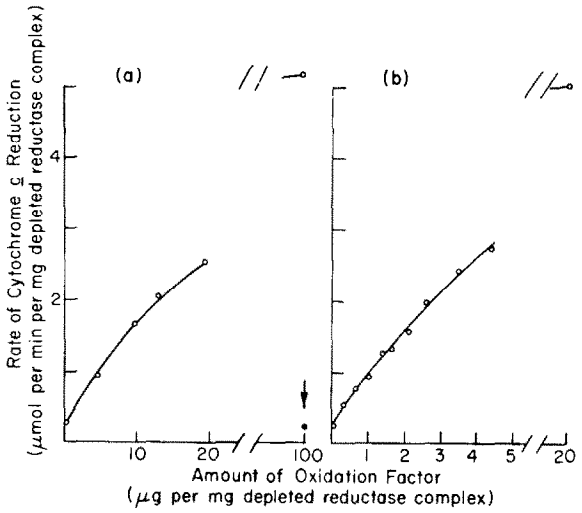

Fig.1. Reconstitution of succinate–cytochrome *c* reductase activity to depleted reductase complex by oxidation factor at two stages of purification. The depleted complex was reconstituted by mixing at 4°C in sequence 20 μl of 200 mM sodium phosphate–200 mM sodium succinate–10 mM EDTA (pH 7.4); 300 μg depleted complex; 20 μl phospholipid-ubiquinone mixture [1]; 65 μg succinate dehydrogenase; and variable amounts of oxidation factor. This mixture was adjusted to 200 μl with H_2O and incubated 60 min at 35°C. The samples were then returned to 4°C and diluted to 2 ml. Curve (a) shows the activity reconstituted by oxidation factor which was partially purified by fractionation with ammonium sulfate. Curve (b) shows the activity reconstituted by oxidation factor which was finally purified by hydroxyapatite chromatography. The arrow points to the activity of the fully reconstituted complex after addition of 2 μg antimycin/mg complex.

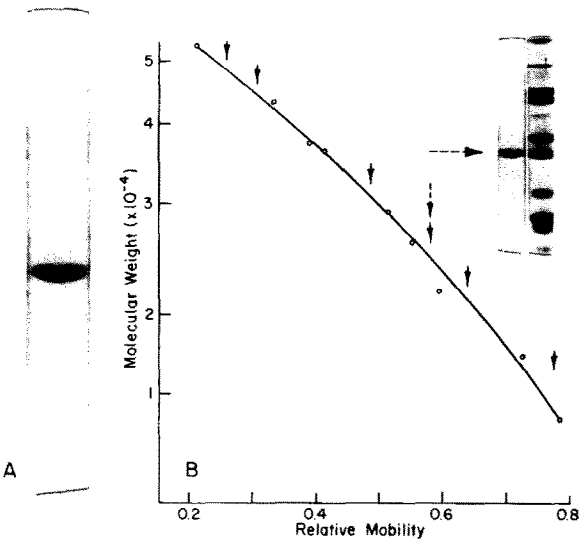

Fig.2. Acrylamide gel electrophoresis [11] of purified oxidation factor. The gel shown in (a) is of the purified protein after hydroxyapatite chromatography. The wire marks the migration position of the tracking dye. The curve in (b) shows the apparent molecular weight of the polypeptides of ubiquinol–cytochrome *c* reductase complex (complex III), indicated by solid arrows, compared to protein standards (open circles). The inset shows electrophoresis gels of oxidation factor and complex III. The dashed arrow shows the relative mobility of oxidation factor, which is identical to the iron–sulfur protein of complex III.

the reconstitution of succinate–cytochrome *c* reductase activity to depleted reductase complex by oxidation factor at two different stages of purity. At low amounts of oxidation factor the restoration of succinate–cytochrome *c* reductase activity is first order with respect to this protein. Thus, under these conditions one can calculate that the partially purified 25-40p fraction (fig.1a) had a specific activity of 144 units/mg. After hydroxyapatite chromatography the activity increases to 690 units/mg (fig.1b) and the protein is homogenous on acrylamide gel electrophoresis in dodecyl sulfate as shown in fig.2a. By way of comparison, the reductase complex which was used as starting material for the purification had oxidation factor activity of 25 units/mg (results not shown).

The purified protein reconstitutes succinate–cytochrome *c* reductase activity of the complex to

Table 1
Properties of reconstitutively active iron-sulfur protein (oxidation factor)

Apparent mol. wt	24 500
Nonheme iron content (nmol/mg)	56
Acid-labile sulfide content (nmol/mg)	36
EPR spectrum (first derivative)	$g_x = 1.78, g_y = 1.90, g_z = 2.02$

The apparent molecular weight was determined by electrophoresis as in fig.2 and the content of nonheme iron and acid-labile sulfide were determined as in section 2. The EPR spectrum was obtained in collaboration with Dr T. Ohnishi

slightly >5 units/mg (fig.1), which is comparable to the activities obtained with the most active preparations of isolated succinate–cytochrome *c* reductase complex [3,12] and the reconstituted activity is fully inhibited by antimycin (fig.1a). These results are evidence that the purified protein possesses the full reconstitutive activity by which oxidation factor was defined [2].

We have identified oxidation factor as a reconstitutively active form of the iron–sulfur protein of the cytochrome b–c_1 segment on the basis of the properties of the purified protein as summarized in table 1. When compared to the polypeptides of isolated ubiquinol–cytochrome *c* reductase complex (complex III) on acrylamide gel electrophoresis in dodecyl sulfate, oxidation factor has the same mobility as the iron–sulfur protein polypeptide of this complex, whose migration position in fig.2b can be established by its location immediately ahead of the M_r 30 600 heme-containing polypeptide of cytochrome c_1 [13,14]. We also mixed oxidation factor with complex III and found that the purified protein and the iron–sulfur protein polypeptide of the complex comigrated as a single, symmetrical band on electrophoresis (results not shown). By this method we obtained 24 500 app. mol. wt for the iron–sulfur protein polypeptide (fig.2b), which is in good agreement with values of 24 000 [15], 24 600 [16] and 25 000 [17,18] obtained by others.

The purified protein contains 56 nmol nonheme iron/mg and 0.65 equiv. acid-labile sulfide/iron. An iron–sulfur protein of the 2 Fe:2 S type, having 24 500 mol. wt, would be expected to have an iron and labile sulfide content of 81–82 nmol/mg. Thus our results indicate that a portion of the iron and labile sulfide has been lost during purification (table 1), which is not uncommon for iron–sulfur proteins [19]. If an intact iron–sulfur cluster is required for reconstitutive activity, as is the case with succinate dehydrogenase [20], the acid-labile sulfide content (table 1) indicates that ~44% of the purified iron–sulfur protein is potentially biologically active. Purified oxidation factor also has an EPR spectrum identical to that of the iron–sulfur protein of complex III [14]. The spectrum is distinguished by a characteristic resonance (first derivative) at $g_y = 1.90$ and resonances at $g_x = 1.78$ and $g_z = 2.02$ (table 1).

The electron transfer activities of the depleted and reconstituted reductase complex are also consistent with the identification of oxidation factor as the iron–sulfur protein of the cytochrome b–c_1 segment. As shown in table 2, the iron–sulfur protein (oxidation factor) is required for succinate–cytochrome *c* reductase and ubiquinol–cytochrome *c* reductase activities, and it is not required for succinate dehydrogenase or succinate–ubiquinone reductase activities. These results agree with work which has shown that the iron–sulfur protein which is present in isolated ubiquinol–cytochrome *c* reductase complex [14] is

Table 2
Electron transfer activities of depleted succinate–cytochrome *c* reductase complex before and after reconstitution with purified iron–sulfur protein

Electron transfer activity	Depleted complex	Reconstituted complex
Succinate dehydrogenase	7.14	8.30
Succinate–ubiquinone reductase	7.59	8.26
Succinate–cytochrome *c* reductase	0.10	5.80
Ubiquinol–cytochrome *c* reductase	0.50	12.0

The depleted complex was incubated with phospholipid, ubiquinone-10, and succinate dehydrogenase as in fig.1. The reconstituted complex was incubated in the same manner, except 25 μg purified iron–sulfur protein, mg complex was included during the incubation. Electron transfer activities were measured as in section 2 and rates are expressed as units/mg

not present in isolated succinate–ubiquinone reductase complex [21,22].

The iron–sulfur protein of the cytochrome b–c_1 segment was first discovered [5]. It had been separated from complex III in an aggregated form and was then solubilized by succinylation and thereafter purified [6]. Thus the purification of oxidation factor represents the first purification of this iron–sulfur protein in a biologically active form. The reconstitution of electron transfer activities by the purified iron–sulfur protein (table 2) demonstrates clearly that it is a required component of the respiratory chain.

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