Determination of molecular mass of the aroid alternative oxidase by radiation-inactivation analysis

Deborah A. BERTHOLD,* Donald J. FLUKE† and James N. SIEDOW*‡
Departments of *Botany and †Zoology, Duke University, Durham, NC 27706, U.S.A.

The functional molecular mass of the cyanide-resistant salicylhydroxamate-sensitive duroquinol oxidase activity from *Symplocarpus foetidus* (skunk cabbage) and *Sauromatum guttatum* spadix mitochondria was determined by radiation-inactivation analysis. The functional molecular mass for the oxidase activity was found to be 26 700 Da for skunk cabbage and 29 700 Da for *Sauromatum guttatum* mitochondria frozen at -70°C . Irradiation of dried mitochondrial samples resulted in a larger target size of 38 000 Da, and in some cases, a stimulation of activity at low dose of radiation. The functional molecular mass of cytochrome *c* oxidase activity from skunk-cabbage and bovine heart mitochondria was also investigated. Dried and frozen mitochondrial samples from both species yielded similar target sizes, in the range 70 900–73 400 Da. Purified bovine heart cytochrome *c* oxidase was also irradiated, and yielded a functional molecular mass of 66 400 Da. The target size of cytochrome *c* oxidase agrees with literature values insofar as the target size is considerably smaller than the molecular mass of the entire complex.

INTRODUCTION

Cyanide-resistant respiration occurs in a variety of plants and fungi, and has been shown to be due to a second or 'alternative' respiratory pathway located in the mitochondrion. The alternative pathway accepts electrons from the ubiquinone pool of the main respiratory pathway and, like cytochrome *c* oxidase in the main pathway, catalyses a four-electron reduction of oxygen to water (Lance *et al.*, 1985; Siedow & Berthold, 1986).

The alternative pathway is believed to consist of a single complex, termed the 'alternative oxidase', which functions as a ubiquinol: oxygen oxidoreductase (Bonner & Rich, 1978). There have been several attempts to purify and characterize the oxidase from aroid spadix mitochondria, where water-soluble ubiquinol analogues can be used to donate electrons directly to the oxidase (Huq & Palmer, 1978; Rich, 1978; Bonner *et al.*, 1986; Elthon & McIntosh, 1986). Because of the apparent lability of the activity of this membrane protein in the presence of detergent, purification has not, to date, been entirely successful. Several reports have, however, suggested that the molecular mass of this enzyme is rather low. For example, genetic evidence from mutants of the fungus *Neurospora crassa* has shown that alternative-oxidase-deficient mutants may lack a 35 kDa band on SDS/polyacrylamide gels that is present in wild-type *Neurospora* (Bertrand *et al.*, 1979). Partial purifications by Bonner *et al.* (1986) and Elthon & McIntosh (1987) likewise have pointed to polypeptide bands on SDS/polyacrylamide gels in the M_r range of 34 000–37 000 as candidates for the oxidase.

Radiation-inactivation analysis, or target theory, is a technique that has proved particularly useful for molecular-mass determinations of membrane-bound proteins. Samples are irradiated with high-energy ioniz-

ing radiation and then assayed for enzyme activity. Single-hit target theory predicts an exponential decay of activity with increasing dose of radiation, and the rate of exponential decay is directly proportional to the mass of the enzyme (Harmon *et al.*, 1985; Kepner & Macey, 1968). One advantage of this technique is that the observed inactivation is independent of other components in the sample, and thus the protein of interest does not need to be pure. Another advantage is that a 'functional' molecular mass is determined; that is, a minimal molecular mass relevant to the activity of the enzyme (Harmon *et al.*, 1985).

In the present study we have used radiation-inactivation analysis to determine a functional molecular mass of the cyanide-resistant quinol oxidase from *Symplocarpus foetidus* (skunk cabbage) and *Sauromatum guttatum* spadix mitochondria. As a control, we also compared the inactivation of skunk-cabbage cytochrome *c* oxidase with the results that we and others have obtained for bovine heart cytochrome *c* oxidase.

MATERIALS AND METHODS

Enzyme and other assays

Cyanide-resistant SHAM-sensitive duroquinol oxidase activity was assayed polarographically at 25°C in a thermostatically controlled cell. The reaction medium was modified from that of Stegink & Siedow (1986) and consisted of 0.7 M-sodium citrate, 0.3 M-mannitol, 10 mM-KCl, 5 mM-MgCl₂, 1 mM-EDTA, 0.1% (w/v) BSA, 250 μM -KCN and 10 mM-potassium phosphate, pH 6.8. High concentrations of citrate were found to stimulate both the skunk-cabbage and *Sauromatum* quinol oxidases, analogously to that reported by Kay & Palmer (1985) for the activity in *Arum maculatum*. Oxygen consumption was initiated upon the addition of 250 μM -

Abbreviations used: BSA, bovine serum albumin; D_{37} , dose of radiation giving 37% survival of activity; E-64, L-trans-epoxysuccinyl-leucylamido-(4-guanidino)butane; SHAM, salicylhydroxamic acid.

‡ To whom requests for reprints should be addressed.

duroquinol, and the non-enzymic oxygen uptake obtained with the addition of 1 mM-SHAM was subtracted to yield a net rate of enzymic oxygen consumption. Cytochrome *c* oxidase (EC 1.9.3.1) was assayed polarographically at 25 °C in 50 mM-potassium phosphate, pH 6.5, containing 0.1 mM-EDTA, 0.1% BSA, 8 mM-sodium ascorbate, 0.5 mM-*NNN'*-tetramethylphenylenediamine, 30 μ M horse heart cytochrome *c* and 1 mM-lauryl maltoside (modified from Suarez *et al.*, 1984).

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) from *Leuconostoc mesenteroides* (Sigma, type XXIV) was assayed spectrophotometrically at 25 °C as NAD⁺ reduction at 340 nm in the presence of 3 mM-NAD⁺, 3 mM-glucose 6-phosphate, 3 mM-MgCl₂ and 50 mM-Tris/HCl, pH 7.8 (Olive & Levy, 1971).

Protein was measured by the method of Lowry *et al.* (1951), with BSA as standard.

Materials

Lauryl maltoside was purchased from Calbiochem-Behring, La Jolla, CA, U.S.A., and duroquinol from K & K Laboratories, Plainview, NY, U.S.A. Horse heart cytochrome *c* was obtained from Sigma, St. Louis, MO, U.S.A., and other chemicals were of the highest grade commercially available.

Mitochondria isolation

Mitochondria from aroid spadix tissue of skunk cabbage and *Sauromatum guttatum* were isolated and purified on sucrose-density gradients as described previously (Stegink & Siedow, 1986), except for the inclusion of 10 μ M-E-64 in the isolation buffers. The purified mitochondria were frozen at -70 °C in a storage buffer consisting of 15% (w/v) sucrose, 10 mM-Tris/HCl, 0.1 mM-EDTA, 0.1% BSA and 10 μ M-E-64, pH 7.2. This storage buffer (with or without E-64) was also used to dilute the mitochondria or the enzymes before irradiation. Purified bovine cytochrome *c* oxidase was irradiated in storage buffer containing 10 mM-lauryl maltoside. Isolated bovine heart mitochondria and purified bovine cytochrome *c* oxidase were given by Dr. G. T. Babcock (Michigan State University, E. Lansing, MI, U.S.A.).

Irradiation procedure

Samples were irradiated in either the dried or the frozen state. Samples were dried for irradiation by a modification of the method of Fluke (1966b). A 25 μ l sample of mitochondria or enzyme to be irradiated was placed on a 2.5 cm square of 125 μ m (0.005 in) Mylar plastic held in good thermal contact with a block of aluminium on ice. A desiccator lid was fitted over the block to connect the samples to a solid-CO₂ trap and a vacuum pump. Twenty samples so treated were degassed by decreasing the pressure incrementally over 1 h, and then the samples were dried in 20 min with a further decrease in pressure. Drying samples in this manner prevented the fluffiness (and concomitant loss of temperature control) associated with more conventional freeze-drying procedures. The dry samples were then transferred to the irradiation chamber, where they were maintained in thermal contact with a block of aluminium held at room temperature. The temperature of the block varied no more than 1 °C over the course of the irradiation.

Samples were frozen for irradiation on a Mylar film held directly on the target block. Irradiation of frozen samples had to be performed at temperatures of -70 °C or lower, to prevent freeze-drying of the sample in the evacuated target chamber.

High-energy ionizing radiation was provided as a 2.1 cm-diameter electron beam by a 1 MeV Van de Graaff accelerator (model J5; High Voltage Engineering Corp, Burlington, MA, U.S.A.) as described previously (Fluke, 1966b). The dose of radiation was calibrated by survival of T₁-bacteriophage plaque-forming ability in *Escherichia coli* B, which can be accurately measured over several orders of magnitude. The *D*₃₇ for the loss of T₁-phage activity was previously determined to be 570 $\pm$ 15 krad, using either γ -rays or several other types of ionizing radiation (Fluke, 1966a).

Samples were either rehydrated or thawed after irradiation and assayed immediately. Resuspension of the dried mitochondrial samples that served as unirradiated controls yielded a 100% recovery of cytochrome *c* oxidase and glucose-6-phosphate dehydrogenase activities, and a 90% recovery of alternative-oxidase activity. Recovery of all activities in unirradiated frozen samples was 100%.

RESULTS

The SHAM-sensitive cyanide-resistant duroquinol oxidase activity in purified skunk-cabbage mitochondria was inactivated exponentially with a dose of low linear-energy-transfer radiation (Fig. 1a), as predicted by single-hit target theory. The empirical relationship of Kepner & Macey (1968) was used to calculate the relative molecular mass (*M*_r) of the target from the experimentally determined dose at which 37% of the initial activity remained (*D*₃₇):

$$M_r = 6.4 \times 10^{11} / D_{37} \quad (\text{in rad})$$

For the data presented in Fig. 1(a), a *D*₃₇ of 44.0 Mrad was obtained. A temperature correction factor of 1.92 for -70 °C was derived from the analysis of Kempner & Haigler (1982), and verified for the alternative oxidase (results not shown). The corrected functional molecular mass for the duroquinol-stimulated alternative oxidase is therefore 27900 Da. Eight independent irradiations of the skunk-cabbage alternative oxidase in frozen mitochondria gave molecular masses in the range 22800–29800 Da, with an average value of 26700 and a standard deviation of 2100. Mitochondria irradiated in either the presence or the absence of the thiol-proteinase inhibitor E-64 yielded similar functional molecular masses. Likewise the inclusion of BSA in the sample buffer up to a concentration of 1.0% had no effect on the observed molecular mass. Irradiation of frozen samples of purified *Sauromatum guttatum* mitochondria gave a similar functional molecular mass of 29700 Da; a representative inactivation is shown in Fig. 1(b).

Irradiation of dried samples of purified skunk-cabbage or *Sauromatum* mitochondria gave a somewhat larger molecular mass of about 38000 Da for seven independent experiments (Berthold *et al.*, 1987). In some experiments a stimulation of activity over that of the unirradiated activity was observed at low dose, resulting in a y-axis intercept at zero dose that ranged from 110% to 180% of the initial activity. A single exponential decay followed the stimulation, yielding the same functional molecular

Fig. 1. Irradiation of aroid alternative oxidase

Frozen samples of skunk-cabbage mitochondria and *Sauromatum guttatum* mitochondria were irradiated at -70°C and assayed as described in the Materials and methods section. (a) Unirradiated skunk-cabbage mitochondria samples (6.1 mg of protein/ml) gave an average rate of duroquinol-stimulated oxygen uptake of 507 nmol of O_2 /min per mg of protein. The D_{37} for this inactivation is 44.0 Mrad. (b) Alternative-oxidase activity in unirradiated frozen samples of *Sauromatum guttatum* mitochondria (1.5 mg of protein/ml) was 3847 nmol of O_2 /min per mg of protein. This inactivation yielded a D_{37} of 41.1 Mrad, and a temperature-corrected molecular mass of 29900 Da. A/A_0 is the fraction of activity remaining after irradiation.

Fig. 2. Irradiation of skunk-cabbage cytochrome *c* oxidase (○) and glucose-6-phosphate dehydrogenase (●)

Frozen samples of skunk-cabbage mitochondria (3.3 mg of protein/ml) were irradiated at -70°C . Cytochrome oxidase activity was assayed as described in the Materials and methods section. Unirradiated samples gave 100% recovery of initial cytochrome *c* oxidase activity, which was 1211 nmol of O_2 /min per mg of protein. The D_{37} for this inactivation was 17.3 Mrad. Dried samples of skunk-cabbage mitochondria containing 15 μg of glucose-6-phosphate dehydrogenase in storage buffer were irradiated at 26°C . The specific activity of glucose-6-phosphate dehydrogenase in unirradiated samples was 362 μmol of NADH/min per mg of protein assayed at 25°C , as described in the Materials and methods section. This inactivation yielded a D_{37} of 5.86 Mrad.

mass of about 38000 Da that was observed with irradiations in which little or no stimulation appeared.

Frozen samples of skunk-cabbage mitochondria were irradiated, and cytochrome *c* oxidase activity was found to be inactivated exponentially with dose of radiation, giving a temperature-corrected functional molecular mass of 70900 Da (Fig. 2). For dried samples of skunk-cabbage mitochondria, the functional molecular mass of cytochrome *c* oxidase was quite similar; two irradiations gave an average value of 71200 Da (Table 1). There was no stimulation of activity at low dose of radiation with cytochrome *c* oxidase, as was found with some inactivations of the alternative oxidase in dried mitochondrial samples.

Cytochrome *c* oxidases purified from plant sources such as pea and sweet potato have been shown to contain only five or six subunits (Matsuoka *et al.*, 1981; Maeshima & Asahi, 1978), in contrast with the eight to 13 subunits commonly observed with mammalian cytochrome *c* oxidase (Hatefi, 1985). In spite of this

difference, the enzymes are similar functionally, and the nucleotide sequences of subunits I and II are highly conserved between the maize, yeast and mammalian oxidases (Isaac *et al.*, 1985; Fox & Leaver, 1981). For this reason the functional molecular mass of skunk-cabbage cytochrome *c* oxidase was compared with that of the bovine heart enzyme.

The inactivation of cytochrome *c* oxidase in bovine heart mitochondria yielded a molecular mass that agreed well with our result from skunk-cabbage mitochondria (Table 1). As with the skunk-cabbage cytochrome *c* oxidase, the values obtained for the functional molecular mass of the bovine enzyme in irradiated mitochondria were similar for dried and frozen samples. Frozen samples of purified enzyme gave a functional molecular mass of 66400 Da when corrected for temperature, which was slightly smaller than, yet consistent with, the results for the membrane-bound enzyme.

Table 1. Functional molecular mass of cytochrome *c* oxidase as determined by radiation inactivation analysis

Cytochrome *c* oxidase was assayed as described in the Materials and methods section. The average initial activity for cytochrome *c* oxidase in skunk-cabbage mitochondria was 730 nmol of O₂/min per mg of protein. The initial activity for cytochrome *c* oxidase in bovine heart mitochondria was 5600 nmol of O₂/min per mg of protein. Purified bovine heart cytochrome *c* oxidase consumed oxygen at a rate of 32.3 μ mol of O₂/min per mg of protein. Temperature correction factors of 1.04 and 1.92 (Kempner & Haigler, 1982) were used to correct the values obtained at 24 °C and -70 °C respectively.

Source	Sample	Irradiation temperature (°C)	Molecular mass (Da)
Spadix			
Mitochondria	Dried	24	71 200
Mitochondria	Frozen	-70	70 900
Heart			
Mitochondria	Dried	24	73 400
Mitochondria	Frozen	-70	71 900
Purified enzyme	Frozen	-70	66 400

Glucose-6-phosphate dehydrogenase has often served as a standard in radiation-inactivation studies (McIntyre & Churchill, 1985; Mandala & Taiz, 1985). To verify further the T₁-phage determination of the dosimetry, glucose-6-phosphate dehydrogenase was added to skunk-cabbage mitochondria before drying and irradiation. Inactivation of a dried sample of glucose-6-phosphate dehydrogenase with dose of ionizing radiation was exponential through three orders of magnitude (Fig. 2). The functional molecular mass derived from these data is 109 200 Da, which is in good agreement with sedimentation-analysis values of 103 600 Da (DiCamelli *et al.*, 1970; Olive & Levy, 1971). Frozen samples of glucose-6-phosphate dehydrogenase irradiated at -70 °C yielded a temperature-corrected molecular mass of 106 700 Da (results not shown).

DISCUSSION

Radiation-inactivation analysis is unique insofar as it allows a determination of the molecular mass of a membrane-associated activity *in situ*. In the present study we have utilized this potential in the determination of the target sizes of two activities in skunk-cabbage mitochondria, the alternative oxidase and cytochrome *c* oxidase. The target size of skunk-cabbage cytochrome *c* oxidase agreed with our determination for bovine heart cytochrome *c* oxidase. The same functional molecular mass of cytochrome *c* oxidase was obtained when either frozen or dried samples were irradiated, a result not always seen with other membrane proteins. Studies using brain membrane opioid receptors (Lai *et al.*, 1984) and neurotoxin receptors (Angelides *et al.*, 1985) have found that the use of freeze-dried samples results in inactivations yielding larger target sizes than when frozen samples were irradiated. Such was also the case when we irradiated dried mitochondrial samples of skunk-cabbage alternative oxidase (Berthold *et al.*, 1987). Because the increased target size occurred only for the alternative oxidase, and not for the endogenous cytochrome *c* oxidase or for the

exogenously added glucose-6-phosphate dehydrogenase, we would conclude that the increased target size for some activities in dried samples is apparently specific to the enzyme activity in question, and not a function of the status of the sample. Regardless of the source of this increased sensitivity to radiation, it is clear that radiation-inactivation studies on membrane-associated proteins need to include control samples in the frozen state.

Some dried mitochondrial samples of the alternative oxidase also yielded a stimulation of activity at low dose of radiation. This effect has been reported previously in other systems (Harmon *et al.*, 1980; Kincaid *et al.*, 1981; Boyer *et al.*, 1986). Two models have been proposed to explain this observed stimulation. One is that a high-molecular-mass inhibitor may be inactivated, allowing a net increase of activity at low dose of radiation (Harmon *et al.*, 1980). The second possibility involves a radiation-induced transition from a low-activity dimeric state to a high-activity monomeric one to explain the observed stimulation (Kincaid *et al.*, 1981). Neither explanation would account for the stimulation observed with the alternative oxidase, because this stimulation is only seen when irradiating dried, but not frozen, samples. We currently have no explanation for this effect.

The functional molecular mass of 66 000–73 000 Da obtained in this study for bovine cytochrome *c* oxidase is consistent with previous work on mammalian cytochrome *c* oxidase, where the functional molecular mass corresponded to an entity much smaller than the entire complex. The molecular mass of mammalian cytochrome *c* oxidase has been reported to be as low as 162 000 Da (Hatefi, 1985) and as high as 203 000 Da (Buse *et al.*, 1985), depending on the assignment of eight or thirteen subunits to the enzyme. In an initial study, Kagawa (1967) reported functional molecular masses of 50 000 and 67 000 Da, respectively, for the mitochondrial and purified cytochrome *c* oxidase activities from rat liver. Thompson *et al.* (1982) observed a value of 70 000 Da for purified bovine cytochrome *c* oxidase. More recently, two separate experimental arrangements yielded functional molecular masses of 114 000 and 99 000 Da for purified bovine heart cytochrome *c* oxidase, and 114 000 Da for the purified rat liver enzyme (Suarez *et al.*, 1984). In all of the above studies, cytochrome *c* oxidase activity was measured polarographically as oxygen consumption. Sone & Kosako (1986) reported a target size of 68 000 Da for purified bovine cytochrome *c* oxidase; however, the activity was measured as a rate of scalar proton uptake. The functional molecular mass of 66 000–73 000 Da obtained in our study for bovine cytochrome *c* oxidase, measured polarographically, compares favourably with these literature values. Radiation-inactivation analysis of cytochrome *c* oxidase in isolated skunk-cabbage mitochondria yielded a functional molecular mass of 71 000 Da, also in accord with the values obtained for bovine cytochrome *c* oxidase.

A functional molecular mass determined by radiation-inactivation analysis may correspond to the molecular mass of a single polypeptide, the sum of the molecular masses of two or more component subunits, or, in some instances, it may represent a somewhat isolated domain of a larger polypeptide (Solomonson *et al.*, 1986, 1987). For an activity associated with diffusible components, as exemplified by the mobile electron-transport carriers in the mitochondrial membrane, the functional molecular mass would correspond to the molecular mass of the

largest component. There is reason to believe that the functional molecular mass of 27000 Da obtained in the present study for the duroquinol oxidase activity of aroid mitochondria corresponds to a single polypeptide. Bertrand *et al.* (1979) have correlated the loss of a 35 kDa polypeptide on an SDS/polyacrylamide gel with the absence of the cyanide-resistant respiratory pathway from mutants of the fungus *Neurospora crassa*. Furthermore, a partial purification of the duroquinol oxidase activity from *Arum maculatum* mitochondria has yielded a major constituent in this same range (30–35 kDa) (Bonner *et al.*, 1986). A partial purification of the alternative oxidase from *Sauromatum guttatum* has also implicated several bands of around 36000 Da as putative oxidase polypeptides (Elthon & McIntosh, 1987). Taking into account both the error associated with radiation-inactivation analysis and the often observed anomalous behaviour of membrane proteins on SDS gels, the value of 27000 Da obtained in this study could correspond to the 30000–35000-Da polypeptide which those groups have implicated in alternative-oxidase activity. However, the possibility of a pathway consisting of freely mobile proteins, of which 27000 Da is the molecular mass of the largest component, cannot strictly be ruled out. It is also possible that the functional molecular mass represents a separate domain or a degradation product of a larger polypeptide. It is clear, however, from the results of this study that the oxygen uptake ascribed to the alternative oxidase is probably not the result of a free-radical oxidation of duroquinol, as proposed by Rustin *et al.* (1984). The association of the SHAM-sensitive duroquinol-dependent oxygen consumption in aroid mitochondria with a molecular mass of 27000 Da argues for the involvement of a protein.

The alternative oxidase, like cytochrome oxidase and laccase, catalyses the four-electron reduction of oxygen to water without any apparent loss of partially reduced oxygen species to the surrounding milieu (Siedow & Berthold, 1986). By analogy with such oxidases, it is likely that the alternative oxidase will contain several metal centres for binding oxygen and accepting reducing equivalents. It must also contain a binding site for the electron donor ubiquinol. The accommodation of these metal centres and binding sites on a functional molecular mass of 27000 Da may prove to be an interesting case of biochemical economy.

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