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Time-resolved study of tryptophan fluorescence in vesicle reconstituted cytochrome oxidase

Effect of redox transition

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Time-resolved study of fluorescence decay of the tryptophan residue in bovine cytochrome *c* oxidase in phospholipid vesicles is reported for the first time. The effect of the redox state of the protein on its conformation has been investigated using time-resolved decay of tryptophan fluorescence in the oxidised and reduced protein. The fluorescence decay was best fitted using a discrete three exponential model. Amplitude distribution of lifetimes also showed three distinct regions in the analysis of decay profiles by the maximum entropy method (MEM). Results of the time resolved studies showed that the amplitudes as well as the lifetimes of the trytophan fluorescence remain the same for the oxidised and the reduced states of cytochrome *c* oxidase, indicating that the environment around tryptophan residues remains more or less unaltered on reduction of the protein. The results suggest that there is no global conformational change in the protein on electron transfer and support the possibility of the existence of local fluctuations in the protein during the redox cycle.

Cytochrome *c* oxidase; Tryptophan fluorescence; Conformational change

1. INTRODUCTION

The complex IV in the respiratory chain consists of the enzyme, cytochrome *c* oxidase (CcO, EC 1.9.3.1) which is involved in the terminal step of electron transfer leading to reduction of dioxygen into water. The enzyme accepts electrons from ferrocytochrome *c* and mediates transmembrane electron transfer from the cytoplasmic side to the matrix side of the inner mitochondrial membrane and reduces dioxygen at the binuclear site [1,2]. The primary electron acceptor in CcO is the Cu_A or heme a site near the cytosolic side and electron transfer takes place to the binuclear heme a_3-Cu_B active center [3,4]. This transmembrane electron transfer has been shown to be coupled with an active proton translocation from inside to outside of the inner mitochondrial membrane [1–3]. It has been proposed that there is a change in the protein conformation at the transition state during this electron transfer process [5]. The nature of this conformational change is a subject of extensive investigation in recent years [6–10].

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Abbreviations: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; NATA, *N*-acetyl tryptophanamide; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; CcO, cytochrome *c* oxidase; COV, vesicle reconstituted CcO; RCR, respiratory control ratio.

The fluorescence of the tryptophan residue has been used to monitor structural changes in various proteins [6–9, 11–16]. In the present report, we have carried out steady-state as well as time-resolved studies of the fluorescence of tryptophan residues of cytochrome *c* oxidase reconstituted in phospholipid vesicles in order to monitor the effect of redox changes on the structure of the protein in vesicular medium.

2. MATERIALS AND METHODS

2.1. *Reagents*

FCCP, soybean phospholipid, cytochrome *c*, valinomycin, NATA, sodium cholate, sodium ascorbate and TMPD were purchased from Sigma. Lauryl maltoside was a product from Fluka. Other reagents used were of purest grade available commercially.

2.2. *Extraction of cytochrome* c *oxidase*

Bovine cytochrome *c* oxidase (CcO) was extracted from beef heart following Yonetani's method [17,18]. The ammonium sulfate fractionation steps were done very carefully and repeatedly to get pure CcO as checked by optical spectra. The concentration was determined by using $\Delta\varepsilon$ (reduced minus oxidised = 25 $mM^{-1} \cdot cm^{-1}$) at 605 nm. Enzymatic activity of CcO was determined by the loss of absorbance of ferrocytochrome *c* monitored at 550 nm [17,18]. The enzyme preparations were stored as small aliquots at −30°C.

2.3. *Preparation of COV*

CcO was reconstituted in soybean phospholipid vesicles by the cholate dialysis procedure [19] at 4°C. The active site orientation was determined by TMPD-induced enhancement of reduction level of COV measured from optical spectra [20,21].

2.4. *Respiratory control ratio (RCR)*

RCR of COV was determined by reported methods [22,23] using FCCP (0.2 μM) and valinomycin (~ 0.02 μM) as uncouplers. The value of RCR of COV varied from preparation to preparation and it decreased on storage of the sample. Only fresh samples of COV with RCR > 2.8 were used for fluorescence studies.

2.5. *Steady-state fluorescence and optical spectroscopy*

Steady-state fluorescence measurements were performed using a Shimadzu RF540 spectrofluorophotometer and optical spectra were recorded by Shimadzu UV-2100 and Cary 17D spectrophotometers.

The fluorescence spectra were corrected for inner filter effect as given by the equation [24], $F_{Corr} = F_{Obs} \cdot 10^{(A_{ex} + A_{em})/2}$. The optimal reductant concentration (0.2–0.3 mM ascorbate/dithionite for COV with 0.8–1.0 μM in CcO) was chosen so that absorbance change due to reductant addition was small and the $10^{(A_{ex} + A_{em})/2}$ term does not have a very significant effect on F_{Corr} in the above equation.

2.6. *Quantum yield*

Quantum yield for the tryptophan fluorescence of COV was determined as described by Hill et al. [25] using NATA in water as a standard and correcting the fluorescence for inner filter effect (see above).

2.7. *Time-resolved fluorescence spectroscopy*

Time-resolved tryptophan fluorescence studies were performed using the tunable picosecond dye-laser pulse from a cavity-dumped dye (Rhodamine 6G) laser driven by synchronously pumped frequency-doubled output (532 nm) of the mode-locked CW Nd-YAG laser system described elsewhere [26]. Fluorescence decay profiles were collected using a time-correlated single photon counting set-up coupled to a microchannel plate photomultiplier [27–29]. The width of the dye-laser pulse was ~ 4 ps and half-width of the instrument response function was typically about 80 ps. The tunable output of the dye laser was further frequency-doubled to generate the second harmonic beam at 292 nm to excite the samples. Emission profiles were collected at the magic angle (54.7°) to eliminate any contribution from anisotropy decay. A Schott WG-320 cut-off filter was used prior to the monochromator, to remove any scattering from vesicles.

Fluorescence decay curves were deconvoluted with the excitation function and the fluorescence intensity was analysed as a sum of discrete exponentials, given by equation 1

$$F = \sum_k A_k \exp(-t/\tau_k) \quad (1)$$

where k is the number of discrete exponentials required to fit the emission profile. To get the amplitudes (A_k) and lifetimes (τ_k), an iterative reconvolution was applied using nonlinear least square fit and Marquardt algorithm for parameter optimization.

Analysis of the fluorescence decay profiles was also carried out using the maximum entropy method (MEM) using 150 lifetime components ranging from 0.01 to 10 ns uniformly distributed in a logarithmic time scale [30,31]. The optimized amplitude distribution ($\alpha(\tau)$) recovered by MEM represent the most probable distribution of amplitudes among the life time components [30,31]. All measurements were done at 24–25°C.

3. RESULTS AND DISCUSSION

The active site orientation of COV was found to have > 80% CcO oriented in the mitochondrial fashion with the heme *a* site residing near the outer side of vesicle. Thus COV represents a realistic model of the mitochondrial complex IV [20,21]. The value of RCR for COV was between 2.8–3.6 (see methods). Steady-state fluorescence of the tryptophan residue of oxidised (native) and reduced COV (0.8–1.0 μM in CcO) was carried out. The quantum yield for tryptophan fluorescence of COV was determined to be 0.046 ± 0.002 (NATA, quantum field = 0.2 [27] in water was used as a standard). An optimal concentration of reducing agent (0.2–0.3 mM $Na_2S_2O_4$ for reducing ~ 1 μM COV) was chosen to reduce COV, so that the absorbance change at 290–350 nm due to the addition of the reductant was < 0.2, and the reduction of the heme *a* site was ensured from the enhancement of the 605 nm band of CcO [20,32].

In the steady-state fluorescence of the tryptophan residue, the observation of a single band (excitation of 292 nm) at 329 nm (Fig. 1) of the oxidised COV indicates that the tryptophan residues are predominantly present in the hydrophobic region in the oxidised state [25]. Addition of reducing agent caused an apparent red shift in the observed fluorescence emission (F_{Obs}) maxima (Fig. 1). The corrected fluorscence spectra (F_{Corr}) obtained by considering the absorptions at the emission and excitation frequencies (inner filter effect) [24] was found, however, to show little effect (Fig. 1) on reduction of the protein. Previous studies in lauryl maltoside solution also indicated the importance of the inner filter effect due to dithionite in the steady-state tryptophan fluorescence of this enzyme [7–9]. Our experiments with CcO in lauryl maltoside using an optimal concentration of the reducing agent to avoid inner filter effect, showed that although the (F_{Obs}) shows a red shift, the (F_{Corr}) of the protein in lauryl maltoside does not change on re-

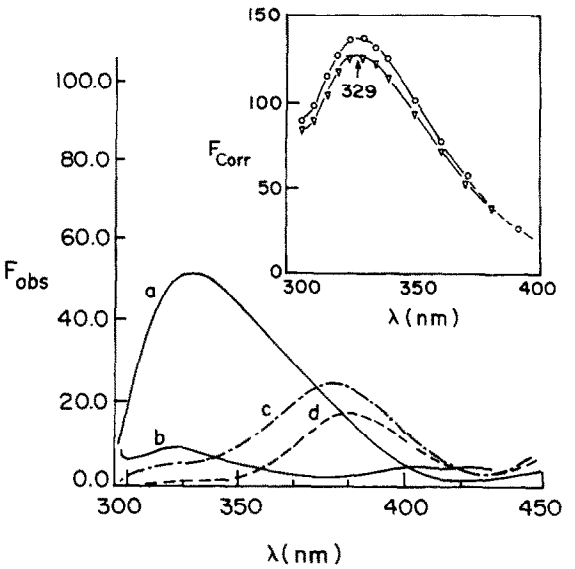

Fig. 1. Fluorescence spectra of cytochrome *c* oxidase vesicles (COV) in oxidised and reduced form. Native COV (~ 1 μM in CcO) in 100 mM KH_2PO_4, pH 7.4 (λ_{ex} = 292 nm) (a) Emission spectra for native COV (oxidised). (b) Background emission intensity due to the blank vesicles prepared by cholate dialysis procedure (see section 2) under similar conditions except without addition of CcO. (c) Reduced COV (0.3 mM in $Na_2S_2O_4$) showing an apparent red shift and drastic quenching of fluorescence intensity. (d) Further addition of reductant (0.5 mM in $Na_2S_2O_4$). Inset are the corrected (see methods) fluorescence spectra of oxidised (○) and reduced (▽) COV showing only little effect on reduction.

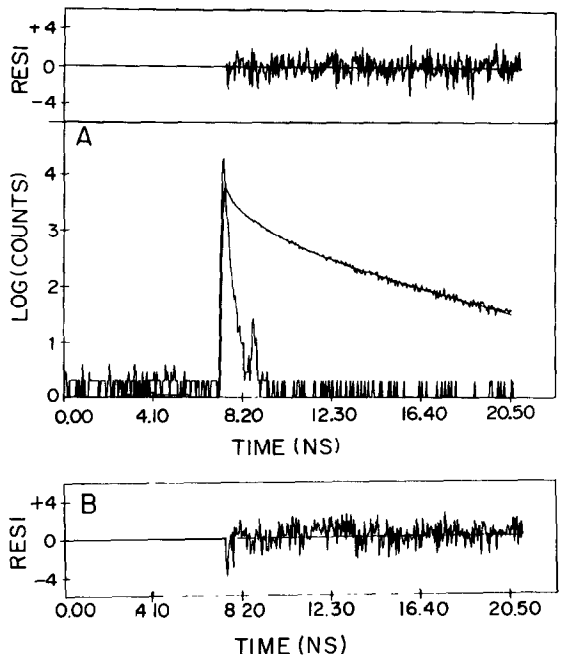

Fig. 2. (A) Time-correlated single photon counting data for tryptophan fluorescence (λ_{ex} = 292 nm and λ_{em} = 330 nm) in vesicle reconstituted cytochrome *c* oxidase (12 μM in CcO) in 100 mM KH_2PO_4, pH 7.4; plot of weighted residuals between theoretical (three-exponential) and experimental emission profiles are shown in top (χ^2 = 1.07, Durbin-Watson parameter = 1.91). (B) Plot of weighted residuals between the tryptophan fluorescence emission profiles of the oxidised protein and that of the reduced (~ 5 mM in $Na_2S_2O_4$) cytochrome *c* oxidase in vesicles.

duction. These results therefore, indicate that an appropriate consideration of the inner filter effect appears to be important in the interpretation of the fluorescence spectra of the tryptophan residue of CcO.

Although an inner filter effect cannot be completely eliminated from the steady-state fluorescence of the tryptophan residue in the present system, the life-times of the fluorescence decay are free from this effect. Thus, a time-resolved study of the decay of tryptophan fluorescence can provide definite information about the effect of redox changes of CcO on the environment around the tryptophan residues in COV. We have studied the decay of tryptophan emission using the picosecond laser setup (see section 2). Fig. 2A shows a representative excitation-emission profile for COV. Time-resolved study was carried out using ~ 5 mM sodium dithionite. The fluorescence decay of tryptophan is known to be highly dependent on the environment and multi-exponential [11,12,14–16,33] behavior have been found in aqueous solutions. However, we have been able to fit satisfactorily the tryptophan decay to a three-exponential model with $\chi^2 < 1.15$. The weighted residuals between the experimental and fitted profile shows (Fig. 2A) the good quality of the fit [27–29]. The time constants for the decay of the tryptophan fluorescence in COV were calculated as: 0.17 ± 0.03, 1.1 ± 0.1 and 4.0 ± 0.1 ns. The corresponding normalized amplitudes were 0.30 ± 0.05, 0.48 ± 0.05 and 0.22 ± 0.02, respectively. Experiments on the oxidised and reduced protein show that the time constants and amplitudes for the decay of tryptophan fluorescence do not change on reduction of the protein.

In order to further check whether or not a three-exponential model is sufficient to analyse the fluorescence of the enzyme, we have carried out a detailed analysis of the decay profiles using the maximum entropy method [30,31]. Fig. 3 shows the results of the MEM analysis of oxidised COV. The MEM simulation results for both the oxidised and reduced COV were also found to give three distinct classes of lifetime distributions with mean lifetimes centered around 0.18, 1.1 and 3.9 ns. The values of the mean lifetimes as well as the general nature of the amplitude distributions remain almost the same in the oxidised and reduced species. The values of lifetimes obtained by a discrete three-exponential model have been plotted (in logarithmic scale) at the top of Fig. 3. The values of mean lifetimes in the MEM analysis match closely with the discrete lifetimes obtained by fitting the experimental data to a three-exponential model.

In order to determine any differences in the fluorescence decay kinetics between the oxidised and reduced species, we have calculated the weighted residuals distribution between the decay profiles obtained from the osidised and reduced COV. Fig. 2B shows a plot of weighted residuals between the emission profile of the oxidised protein and that of the reduced protein in vesicles. A near-random distribution of weighted residuals [28,29] in this case further indicates that the tryptophan fluorescence decay may not be affected by the reduction of the protein. The circular dichroism spectra of the oxidised and reduced protein also did not show any change in the 250–190 nm region indicating that there is no change in the bulk conformation of the protein on reduction.

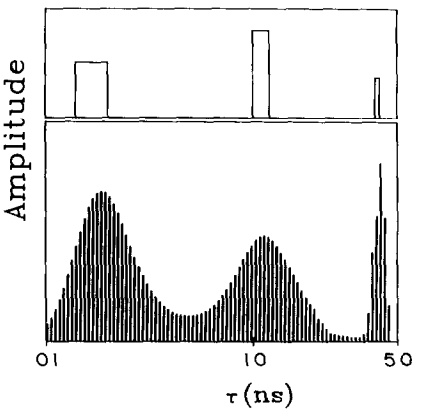

Fig. 3. Amplitude profile of fluorescence lifetimes (in logarithmic scale) of oxidised COV recovered by MEM (χ^2 = 1.2). The upper plot shows the result of discrete three exponential fit to the data with normalized amplitudes and with width representing uncertainty in the lifetime values.

The fluorescence lifetimes of COV treated with ferricytochrome *c* were found to show an overall decrease (~ 5%), indicating that the interactions of cytochrome *c* with cytochrome *c* oxidase might be causing some change in the protein conformation affecting tryptophan fluorescence. However, addition of the reducing agent to the cytochrome *c*–COV system does not show any further change in the fluorescence life times.

In order to further check the sensitivity of the tryptophan fluorescence decay parameters on the structural changes of the protein, we have studied the denatured protein in vesicles. The tryptophan fluorescence for the denatured cytochrome *c* oxidase in vesicles could not be fitted to three exponentials. This is indicative of a heterogeneous distribution of the tryptophan residues in the denatured protein causing large variations in the environment around the tryptophan residues.

4. CONCLUSIONS

The present results show that the tryptophan steady-state fluorescence as well as decay times do not change on reduction of cytochrome *c* oxidase in vesicles. Therefore, the transmembrane electron transfer in the protein may not be associated with any gross structural change which otherwise would have affected the environment of the tryptophan residues. It has been shown previously that the redox changes in cytochrome *c* oxidase may not need any large scale structural variations [8]. Our results also indicate that the transmembrane electron transfer coupled with a proton pump may not involve any global change in conformation. The structural rearrangements associated with localized fluctuations in the protein backbone and/or in the amino acid side chains [9] may possibly be involved in the redox changes of the protein and such rearrangements in the protein structure may not cause changes in the environment of the tryptophan residues.

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