Minireview

Some recent contributions of FTIR difference spectroscopy to the study of cytochrome oxidase[1]

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Abstract In this minireview, some of the new findings using infrared spectroscopy to study cytochrome oxidase will be reviewed, with an emphasis on those studies involving our laboratory.

Key words: FTIR; Oxidase; Proton; Spectroscopy

1. What can infrared spectroscopy tell us?

FTIR (Fourier transform infrared) spectroscopy is a standard procedure in the characterization of small molecules. Modern commercial instrumentation has exquisite signal/noise, and can record changes at the level of a single amino acid residue, but despite this, the applications to exploit this capability using biochemical systems are much more limited [1]. There are good reasons why this is the case. One important reason is that virtually any biochemical study uses water as a solvent, and H_2O absorbs IR light in the region of greatest interest for protein studies. This problem can be solved, or at least evaded, under favorable circumstances, for example, by using high protein concentrations and a very short path length cell and by using D_2O instead of H_2O.

Another major problem is that the FTIR spectrum of a protein will have a very large number of absorption bands from the vibrational modes of all the backbone amides and all of the side chains. The vibrational frequencies of these absorption bands depend on hydrogen bonding, protonation state and other structural considerations. In favorable cases, this problem can also be solved by using difference spectroscopy (see below) and by using isotopes and/or mutants to remove or shift contributions from particular groups. In practice, FTIR spectroscopy has proved to be particularly valuable (though certainly not limited to) observing changes in either the protonation or hydrogen bonding of carboxyl groups (COOH), i.e. aspartates and glutamates, because the extinction coefficient of the C=O stretching mode is relatively high and because there bands are relatively isolated in the spectrum. Cytochrome oxidase is well suited for studies by FTIR spectroscopy [2–14]. The technique requires small amounts of protein (typically a few μl of 300 μM protein) as a practical consideration and provides information that is essential to characterize the molecular mechanism. Consider the challenge to monitor the vibrational absorption bands of a single glutamic acid COOH group in a protein which contains on the order of 10^3 residues. The difficulties are obviously substantial. In practice, this amounts to being able to monitor an absorption of about 10^{-5} OD in a background of about 1 OD. This demands very low random noise and drift (1 part in 10^{-6}). This is best achieved by the use of difference spectroscopy; the FTIR spectrum of a single sample is recorded in two different states of the protein, and these spectra are then subtracted. Only those components of the spectra that are different in the two states will give non-zero components. However, because the changes are so small, it is necessary to alter the state of the sample while it is in the spectrophotometer cell, thus avoiding physical perturbations (e.g. sample cell alignment shifts) that could easily wipe out any traces of the small spectroscopic changes due to the protein structural perturbation.

Several approaches have been exploited to obtain difference FTIR spectra of proteins, and all have been applied to cytochrome oxidase. The goal in all cases is to maintain the sample at a constant concentration and path length, while perturbing the state of the protein in a way that is informative.

2. Light-activated processes

The FTIR spectrum of the sample is taken prior to and after (or during) illumination. If the process is a reversible photo-cycle, then the changes in the FTIR spectrum can be time-resolved by repetitive flashing and signal averaging. Much of the technical development of FTIR as applied to biochemical problems has involved photo-reactive systems, in particular bacteriorhodopsin [1,15–17] and the photosynthetic reaction center [18,19]. Cytochrome oxidase can be reduced in situ by photoreducing agents [20] or oxidized using 'caged O_2' [4,20]. Also, the response of the enzyme to photodissociation of CO can be measured upon laser photolysis of the CO adduct of either the fully reduced enzyme or the two-electron reduced (mixed valence) form [3,5,6,10,12–14,21–23].

3. Electrochemical changes

The FTIR sample cell can be equipped with electrodes to

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control the electrochemical solution potential. FTIR spectra are then recorded as the enzyme is reduced/oxidized [9,24].

4. Perfusion-induced processes

This requires the use of a technique called attenuated total reflectance (ATR) FTIR spectroscopy [25]. The protein is placed on the surface of a crystal (e.g. diamond or silicon) as a thin film. The IR light is passed through the crystal, reflecting off the surface with which the protein is in contact. At the points where the light is reflected, the radiation penetrates (an evanescent wave) into the sample to a depth of the order of 1 μm (wavelength-dependent). Membrane proteins containing lipids have been found to form stable films after briefly being air-dried and then re-hydrated. Buffer can be flowed over the film and the medium to which the protein is exposed can, therefore, be changed by perfusion. Cytochrome oxidase can be reduced by dithionite, for example, or reacted with hydrogen peroxide to form an oxygenated state equivalent to an intermediate in the catalytic cycle [3,4,26–28].

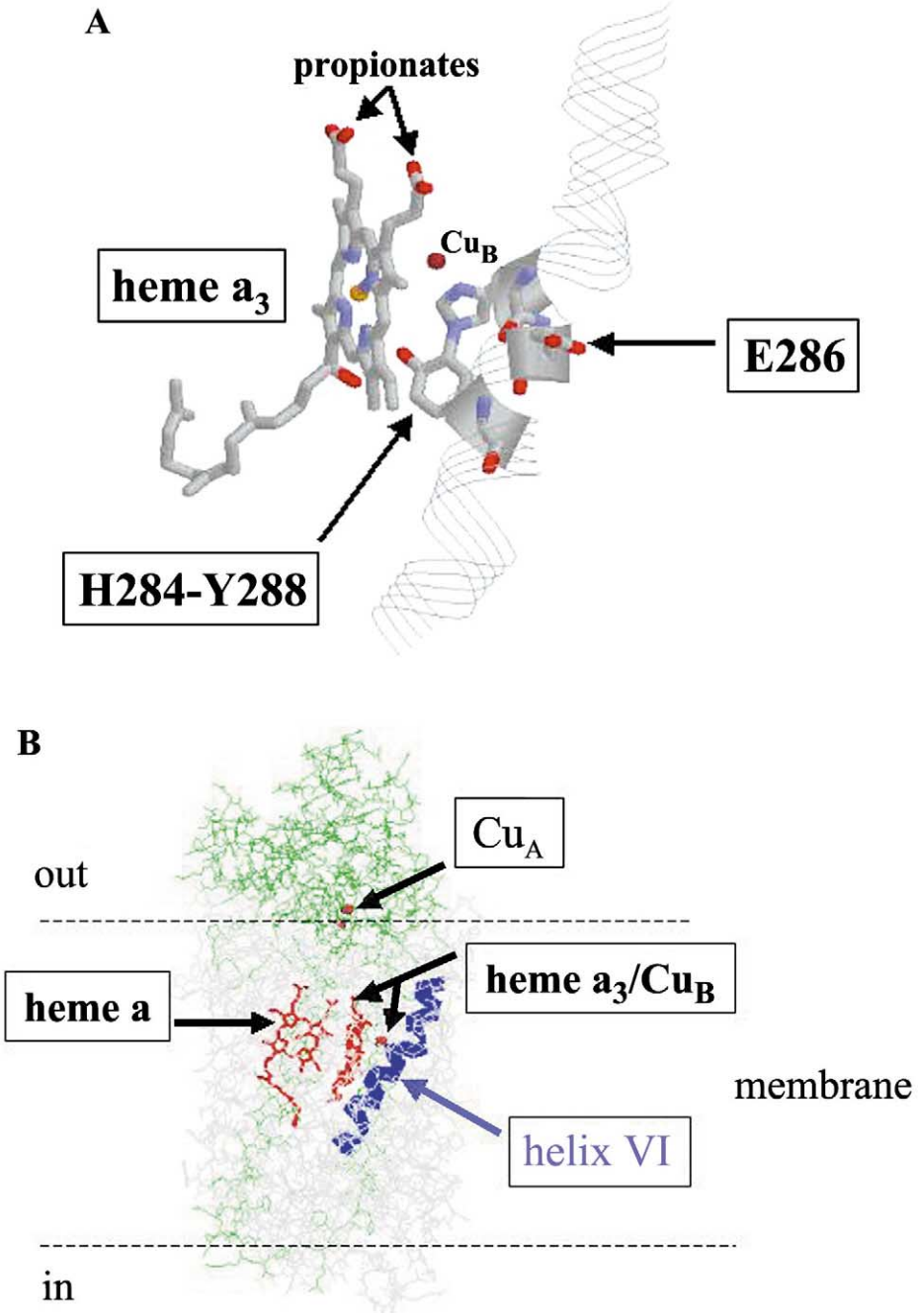

Fig. 1. Structure of *R. sphaeroides* cytochrome c oxidase (see [30]). Panel B shows a large view with subunits I and II. The four redox-active metal centers are shown in relationship to the protein, which measures about 90 Å from top to bottom. Also shown is a portion of transmembrane helix VI (residues 275–295) in blue. This portion of the protein contains E286 and H284–Y288 which are key to the function of the oxidase, and which are observed by FTIR spectroscopy. Panel A shows a close view of the heme a_3/Cu_B active site with E286 and the cross-linked H284–Y288 pair shown. The heme a_3 propionates are also shown since these have also been implicated in proton pumping and can be observed by FTIR spectroscopy [9,40].

5. Cytochrome oxidase

During each catalytic cycle of cytochrome oxidase, one molecule of O_2 is reduced to two H_2O molecules. The active site is buried deep within the protein [29]. The protons that are delivered to the active site must be translocated about 30 Å from the bacterial cytoplasm (or mitochondrial matrix for the eukaryotic enzyme). In addition to the four protons utilized to make two H_2O, another four protons are translocated all the way across the membrane through the protein, i.e. pumped. The proton and electron transfer reactions are strongly coupled. One element of understanding how the enzyme functions is to determine which groups are protonated and when. FTIR difference spectroscopy has the potential to provide definitive answers to these questions.

6. The active site

Cytochrome oxidase contains four redox-active metal centers: Cu_A (a two-copper center), heme a, heme a_3 and Cu_B. Heme a_3 and Cu_B constitute the heme/copper active site where O_2 binds (to ferrous heme a_3) and is reduced to water. Fig. 1 shows the active site of the cytochrome c oxidase from *Rhodobacter sphaeroides* [30]. Note the unique cross-linked pair of amino acids H284–Y288 (Fig. 1).

7. The catalytic cycle [31,32]

Fig. 2 shows a schematic of the catalytic cycle, with several intermediates defined by the status of the heme/copper center.

1. O: The oxidized state of the enzyme, in which all four metal centers are oxidized.
2. E: The one-electron reduced heme/copper center.
3. R_2: The two-electron reduced binuclear center, which reacts with O_2.
4. P_m: Product of the reaction of O_2 with the two-electron reduced enzyme (R_2). The O–O bond is cleaved in this state. This requires four electrons, since the valence of each oxygen atom is reduced from 0 to −2. One electron comes from $Cu_B^+ \rightarrow Cu_B^{2+}$; two electrons come from the heme a_3 iron, $Fe^{2+} \rightarrow Fe^{4+}$; and it is postulated that the fourth electron comes from the active-site tyrosine in H284–Y288, which forms a neutral radical. One of the oxygen atoms is associated with the ferryl state of heme a_3 ($Fe^{4+}=O^{2-}$) and the other is associated with $Cu_B^{2+}(^-OH)$.
5. F: The presumed tyrosyl radical is reduced back to tyrosine (or tyrosinate). The addition of another 'fourth' electron reduces heme a_3 from the Fe^{4+} state back to ferric Fe^{3+}, and state O of the enzyme.

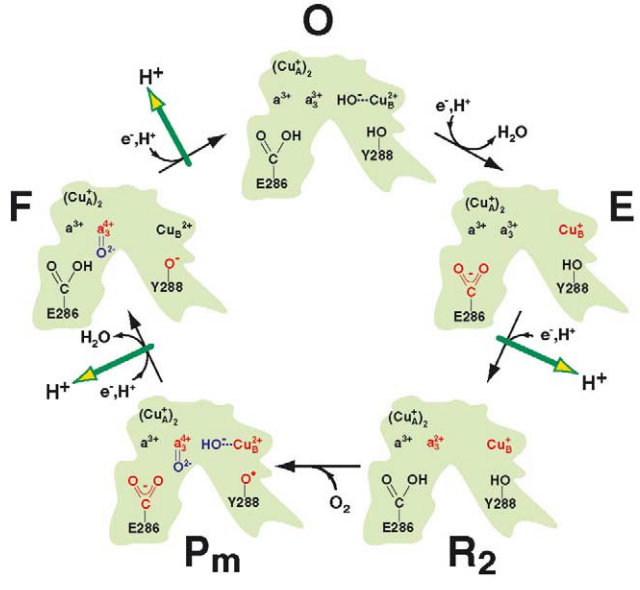

Fig. 2. Schematic showing the catalytic cycle of cytochrome oxidase. The protonation states of E286 and the H284–Y288 tyrosine from the *R. sphaeroides* oxidase, as determined by Nyquist et al. [3], are shown. The figure is taken from [3].

8. Proton pumping [33,24]

For each cycle ($O_2 \rightarrow 2H_2O$), the enzyme pumps four protons across the membrane. A total of eight protons are taken up from the negative side of the membrane (bacterial cytoplasm or mitochondrial matrix). Four of these protons are consumed to make two H_2O at the active site, and another four protons are pumped to the positive side of the membrane (bacterial periplasm or mitochondrial intermembrane space). The steps coupled to the proton pump are a matter of debate [33–39]. There is an average of one H^+ pumped per electron. It is certain that $P_m \rightarrow F$ and $F \rightarrow O$ are coupled to proton pumping, and recent evidence suggests that the 'reductive phase' $O \rightarrow E \rightarrow R_2$ may also be coupled to pumping [34,37]. It is far from settled, but it is possible that each of the four electron transfer reactions from heme a to the heme a_3/Cu_B center is coupled to pumping one proton across the membrane.

9. Strategies for using FTIR spectroscopy

FTIR spectroscopy has the potential to report the protonation changes within the enzyme during any one of the steps (e.g. $F \rightarrow O$). Current applications of FTIR are, however, far from this goal, but are establishing the basic information that will be needed to do the time-resolved experiments. Most of the work to date has involved obtaining static difference spectra between various forms of the enzyme. The difficulties of assignment and interpretation of the spectra are substantial because of the overlap of the many vibrational absorption bands. Use of bacterial oxidases is essential to enable the comparisons of site-directed mutants with the wild-type enzymes, and also to enable the incorporation of isotopic labels that will selectively shift vibrational features of bands in a predictable manner. Some examples of recently published work are summarized below.

9.1. Photolysis of the CO adduct of the fully reduced (R_4) oxidase (Spectrum R_4–R_4CO)

Upon photolysis of the CO adduct of the fully reduced bovine oxidase, time-resolved FTIR shows that the CO moves from heme a_3 to Cu_B, a distance of about 5 Å, in less than 1 ps [21]. After about 2 μs, the CO dissociates from Cu_B and equilibrates with the bulk solution [5]. The rate of recombination of CO from bulk solution to heme a_3 is about 10 ms at 1 mM CO. The time-resolved FTIR shows, furthermore, that there are changes in the region of the spectrum where COOH groups from aspartate and glutamate absorb [5]. An absorp-

tion band assigned to E242 (equivalent to E286 in Fig. 1) shifts from 1741 to 1750 cm^{-1} (in H_2O buffer) as CO is dissociated from the fully reduced enzyme to form the unliganded state. The spectroscopic shift suggests a change in hydrogen bonding of the C=O bond of E242, and the dynamics of this process coincides with the dissociation of CO from Cu_B [5]. A linkage between the ligation state of Cu_B and the hydrogen bonding to E286 is also observed in studies of cytochrome bo_3 from *Escherichia coli* [13]. Most importantly, these studies show that E286 is protonated in the fully reduced enzyme, up to pH 9. In both the bovine and *E. coli* oxidases, there is evidence for the perturbation of at least one additional COOH group upon photolysis of CO. Surprisingly, CO photolysis from the fully reduced oxidase does not result in changes in the E286 environment of either the *Paracoccus denitrificans* [10] or the *R. sphaeroides* [5] oxidases.

Other residues in the vicinity of the active site have also been shown to be perturbed upon photodissociation of CO from the fully reduced enzyme. In a recent study of the oxidase from *Thermus thermophilus*, a perturabation of the COOH group of one of the heme a_3 propionates was observed upon photolysis of CO, coincident with decay of the transient CO–Cu_B adduct [40]. Photodissociation of CO from the reduced *E. coli* oxidase has been shown to perturb the H284–Y288 cross-linked residues at the enzyme active site [14]. In summary, simple ligand changes at the heme/copper metal centers cause structural rearrangements in the vicinity of heme a_3 and Cu_B.

9.2. *Fully reduced-minus-oxidized enzyme (Spectrum R_4–O)*

Full reduction of the oxidases from bovine [26], *R. sphaeroides* [4], *P. denitrificans* [11,24] and *E. coli* [20] results in changing the hydrogen bonding environment of E286 (Fig. 1). The absorption at around 1735 cm^{-1} shifts to 1745 cm^{-1} upon full reduction of the *R. sphaeroides* oxidase [4], for example (Fig. 3). The derivative shape results from the subtraction of the 1745 cm^{-1} band from the 1735 cm^{-1} band. The assignment of this spectral feature was accomplished by comparing the FTIR difference spectrum of the E286D mutant, in which the features are shifted to 1739 and 1729 cm^{-1}. The positions of the bands are also shifted by performing the experiment in D_2O, due to the change from COOH to COOD. The same results are obtained over the pH range from 5.0 to 9.5, showing that this buried glutamic acid remains protonated up to pH 9.5 in both the fully oxidized and fully reduced forms of the enzyme. Work on the *E. coli* oxidase has demonstrated that the change in the environment of E286 is due to the reduction/oxidation of the low-spin heme (heme a for the *R. sphaeroides* oxidase) and not the redox state of the heme/copper center [20]. The equivalent experiment with the bovine oxidase shows perturbations to another carboxyl group, which appears to be deprotonated upon full reduction of the enzyme [6,26]. Based on the changes observed in the X-ray structure upon reduction of the enzyme [41], it has been concluded that the spectroscopic feature is most likely due to deprotonation of residue D51 upon reduction of the enzyme. The bacterial oxidases do not have the equivalent of this residue.

9.3. *One-electron reduced binuclear center-minus-CO adduct of mixed valence enzyme (Spectrum E–R_2CO)*

The CO adduct of the two-electron reduced enzyme (R_2CO) has both electrons in the heme/copper center while heme a and Cu_A are oxidized. Upon photolysis of CO, the reduced form of heme a_3 is destabilized and the result is 'back' electron flow from heme a_3 to heme a. Hence, there is a transient species formed in which heme a and Cu_B are reduced while heme a_3 and Cu_A are oxidized. This species, with a one-electron reduced heme/copper center, is called species E in this work. This species is a reasonable mimic of an intermediate expected during catalysis. The E–R_2CO FTIR difference spectrum of the *R. sphaeroides* oxidase [3] shows a trough at 1745 cm^{-1} (Fig. 3). This suggests that the glutamate COOH is deprotonated to COO^-; the deprotonated form does not absorb in the same region of the spectrum. Comparison with the E286D mutant confirms that this trough is due to E286, and the simplest explanation is that E286 is deprotonated in the E state. Similar studies on the *P. denitrificans* enzyme show a perturbation of E286 which cannot be clearly interpreted as a deprotonation but may be an environmental change [8].

Two studies [12,42] of the bovine oxidase also show evi-

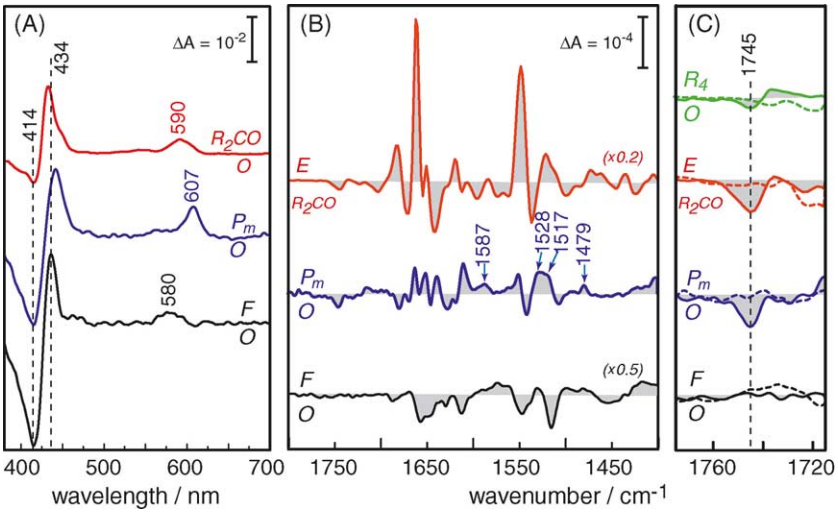

Fig. 3. FTIR difference spectra of the *R. sphaeroides* oxidase, showing changes in structure as the state of the enzyme is altered. Panel A shows the UV–vis spectra of the protein in the various states, and panels B and C show the FTIR difference spectra. The figure is taken from [3].

dence of deprotonation of an acidic residue upon back electron transfer from heme a_3 to heme a. In the study by McMahon et al. [12], the E–R_2CO difference spectrum was interpreted by analogy to the work described above on the *R. sphaeroides* oxidase [3]. It was, therefore, concluded that E242 (equivalent to E286 in *R. sphaeroides*) is deprotonated upon back electron transfer. The work reported by Okuno et al. [6] showed that an acidic residue was deprotonating upon back electron flow in the bovine enzyme. It was concluded that the deprotoning residue in the bovine oxidase is likely D51, consistent with the interpretation that D51 is deprotonated in the fully reduced-minus-oxidized spectrum of the bovine enzyme [6]. Further work will be needed to clarify which interpretation is correct.

9.4. Oxygenated P_m state-minus-oxidized enzyme (Spectrum P_m–O)

When the two-electron reduced enzyme is reacted with O_2 it forms an oxygenated state that is identical or very similar to a catalytic intermediate. Using perfusion-induced FTIR spectroscopy, two groups have reported P_m–O FTIR difference spectra [3,27,28]. The results obtained by Nyquist et al. [3] with the *R. sphaeroides* oxidase indicate a trough at 1745 cm^{-1} without a significant positive feature. It has been concluded that E286 is deprotonated in the P_m state of the enzyme (Fig. 3). Comparison with E286D confirmed that E286 is the source of the absorption change (Fig. 3). In addition, there are positive bands at 1587, 1528 and 1517 cm^{-1} that could originate from a tyrosyl radical form of H284–Y286. Iwaki et al. [28] studied both the bovine and the *P. denitrificans* oxidases. Their data show a strong derivative-shaped feature in the spectrum of the *P. denitrificans* oxidase that is interpreted as a perturbation, but not deprotonation of E278 (E286 equivalent in *P. denitrificans*). The spectrum of the bovine oxidase also shows multiple features in the same region of the spectrum, which could represent either deprotonation or perturbation of the protonated COOH species. Several peaks are suggested as possibly originating from the formation of a tyrosyl radical at the histidine–tyrosine pair but, as with the spectrum of the *R. sphaeroides* oxidase, the assignments are very tentative.

9.5. Oxygenated F state-minus-oxidized enzyme (Spectrum F–O)

By treating the oxidase with H_2O_2, the majority of the enzyme can be converted to the F state, very similar or identical to the species that is a catalytic intermediate. The F–O FTIR difference spectra of the *R. sphaeroides* [3], *P. denitrificans* [28] and bovine oxidases [28] have been reported. The spectroscopic changes are small compared to the P_m–O spectrum in all cases, and there is no perturbation observed in the 1745 cm^{-1} region. The F–O spectrum of the *R. sphaeroides* oxidase has several features that suggest that the histidine–tyrosine pair is perturbed and probably deprotonated to form a tyrosinate [3]. Hence, the data support the expectation that the presumed tyrosyl radical in the P_m state is reduced upon formation of the F state. The spectra of the *P. denitrificans* and bovine oxidases have also been interpreted to indicate reduction of the tyrosyl radical, but that the protonated tyrosine is present in state F [28]. The spectra in all cases suggest that the reduction of the tyrosyl radical brings the enzyme to a closer approximation of the oxidized enzyme, eliminating many of the structural perturbations induced upon formation of the P_m state.

10. Summary

FTIR spectroscopy is already proving to be of great value in the study of cytochrome oxidase. Although the different groups participaing in these studies are not in full agreement, there is much that is common and tools are available to resolve the experimental and interpretational differences. The method provides a unique experimental window into the heart of the oxidase and will allow us to eventually observe the sequence of proton movements that constitute the proton pump.

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