Subunit change in cytochrome c oxidase: identification of the oxygen switch in *Dictyostelium*

Roberto Bisson¹, Silvia Vettore,
Elisabetta Aratri and Dorianna Sandonà

Centro CNR Biomembrane and Dipartimento di Scienze Biomediche
Sperimentali, via G. Colombo 3, 35121 Padova, Italy

¹Corresponding author

Cytochrome c oxidase (COX) has a complex modular structure in eukaryotes. Depending on growth conditions, interchangeable isoforms of selected subunits are synthesized and combined to the evolutionarily conserved catalytic core of the enzyme. In *Dictyostelium* this structural make-up is regulated by oxygen and involves two forms of the smallest subunit, termed VIIe and VIIs. Here we show that, in spite of a considerable sequence divergency, they are encoded by adjacent genes, linked 'tail to head' by only 800 bp. Deletion analyses reveal the presence of a short intergenic segment acting as an oxygen transcriptional switch. This structural organization and the different stability of the two subunit isoforms offer a molecular explanation for the extraordinary sensitivity to oxygen of the switching mechanism.

Keywords: cytochrome c oxidase/*Dictyostelium*/oxygen/
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Introduction

Essentially all living organisms have evolved mechanisms to adapt their metabolism to environmental changes. A striking example is offered by the energy-generating system of many prokaryotes where entirely different protein complexes, synthesized in response to fluctuations in the supply of specific substrates, constitute the flexible respiratory network that allows survival in fast-changing environments (Van Spanning *et al.*, 1995). This ability has been drastically reduced in eukaryotes and predominantly restricted to the terminal part of the respiratory chain. Moreover, though in some microorganisms and plant tissues two alternative oxygen-reducing enzymes can still be found (McIntosh, 1994), a different adaptive strategy, based on limited subunit changes around an invariant catalytic core, was evolved and eventually prevailed in animal cells (Kadenbach *et al.*, 1987).

This modular complex is cytochrome c oxidase (COX), the integral membrane protein that in mitochondria and many aerobic bacteria catalyses electron transfer from cytochrome c to molecular oxygen coupled to proton translocation (Capaldi, 1990; Babcock and Wikstrom, 1992). Recently, its crystal structure from *Paracoccus denitrificans* (Iwata *et al.*, 1995) and from bovine heart (Tsukihara *et al.*, 1996) at 2.8 Å resolution has been published. The enzyme catalytic core is constituted by the

two largest subunits that contain three redox centres (cytochrome *a*, Cu_A, and the binuclear cytochrome *a*₃-Cu_B site). With a third subunit, they represent the typical structure of the bacterial complex that has been conserved throughout evolution (Castresana *et al.*, 1994). In eukaryotes, they are encoded by the mitochondrial genome and are assembled with 4–10 different smaller subunits coded for in the nucleus (Capaldi, 1990). As mentioned, some of these additional components have alternative forms that are tissue specific or developmentally regulated in higher organisms and environmentally controlled in lower eukaryotes (Kadenbach *et al.*, 1987; Hodge *et al.*, 1989; Schiavo and Bisson, 1989; Parson *et al.*, 1996). The nuclear subunits could therefore act as regulators of the enzyme activity either directly or via binding of allosteric effectors. Recent findings add support to both possibilities. In yeast, the switching between two subunit isoforms affects the binuclear reaction centre and alters the kinetics of interaction with cytochrome c (Allen *et al.*, 1995). In mammals, under physiological concentration, ATP allosterically influences the enzyme kinetics by differential binding to the liver and heart isoforms of the same nuclear subunit (Anthony *et al.*, 1993; Frank and Kadenbach, 1996).

The *in vivo* physiological meaning of these observations is a matter of debate not only for the general understanding of the structure–function relationship of the key enzyme of the aerobic metabolism, but also for the implications concerning an emerging group of mitochondrial diseases involving oxidative phosphorylation defects (Wallace, 1993; Brown and Wallace, 1994; Kadenbach *et al.*, 1995; Tiranti *et al.*, 1995). In this respect, the selection of suitable model systems is an important aspect of these studies.

We are investigating these issues in the slime mould *Dictyostelium discoideum* because of some interesting features that characterize its lifestyle and the enzyme structure. This strictly aerobic organism lives in the forest soil as single cell amoeba, feeding on bacteria and other nutrients from decaying leaves. Upon starvation, cells can aggregate and differentiate in two major cell types that, eventually, after a 24 h developmental stage, form a cellulose stalk holding on top a balloon-like structure filled with spores (Loomis, 1982). The process shows an extraordinary sensitivity to oxygen that also affects the relatively simple subunit composition of COX (Sandonà *et al.*, 1995). A 20–30% decrease of the oxygen tension is in fact sufficient to trigger the expression of an alternative form of subunit VII, the smallest polypeptide of the enzyme, which exclusively prevails under hypoxia (Schiavo and Bisson, 1989).

In order to investigate the molecular mechanism involved in the oxygen-dependent, highly coordinated expression of the two subunit isoforms (termed VIIe and VIIs), we have cloned and characterized the genes. They are located in a short segment of genomic DNA, in a tail-

to-head array. This organization, which is unique among the COX isogenes of other eukaryotic organisms, appears related to the presence of a common *cis*-active oxygen-responsive element(s) located in the middle of the intergenic region. Though expression is regulated at the transcriptional level, we show that the different efficiency in enzyme assembly of the two subunit isoforms, a possible consequence of their diverse stability, has considerably increased the sensitivity of the cell response under conditions of mild hypoxia.

Results

Characterization of the two genes encoding alternative forms of COX subunit VII

As isolated, *Dictyostelium* COX comprises six polypeptides, usually identified by roman numerals. Subunits I and II are the largest mitochondrially encoded subunits, while subunits IV, V, VI and VII are the products of nuclear genes. A third mitochondrial component, subunit III, which is common to all eukaryotic and most bacterial COX, is lost during purification (Bisson *et al.*, 1985). Subunit VIIe is the smallest subunit that is substituted by an alternative isoform, termed VIIs, under hypoxia. A subunit VIIe cDNA (Rizzuto *et al.*, 1990) and a subunit VIIs gene fragment obtained by PCR (Sandonà *et al.*, 1995) were used for restriction analysis of the genes. Southern blot hybridization of the genomic DNA, after digestion with appropriate restriction enzymes (Figure 1A), shows that they are present as a single copy. Thus, in confirmation of a previous observation (Rizzuto *et al.*, 1993), the nuclear genes of *Dictyostelium* COX do not share the complexity found in mammals, characterized by the existence of dispersed multigene families with a number of pseudogenes (Suske *et al.*, 1987; Carrero-Valenzuela *et al.*, 1991; Taanman *et al.*, 1991; Arnaudo *et al.*, 1992; Seelan and Grossman, 1993; Mell *et al.*, 1994). In this connection, an additional unprecedented feature, immediately evident from the identical position of several restriction fragments after re-hybridization of the filter with the alternative probe, is the close proximity of the two genes. As shown by the restriction map of Figure 1B, they are ~800 bp apart and, as later confirmed by sequencing, in a 'tail-to-head' configuration.

The 4.9 kb *Bgl*III restriction fragment (Figure 1A) was partially purified by fractionation in agarose gel, inserted into the *Bam*HI site of a pUC19 vector and cloned as detailed in Materials and methods. A set of deleted subclones, extending either 5' or 3' of the DNA region (Figure 1B), were then generated by a nested deletion system based on exonuclease III/mung bean nuclease degradation of DNA and used for sequencing and analysis of gene expression.

The presence in the cloned region of the *cis*-active sequences needed for oxygen regulation was directly tested in *Dictyostelium* stable transfectants obtained by electroporation (Rizzuto *et al.*, 1993), in the presence of the plasmid containing the *Bgl*III fragment and selection. Southern blotting analysis of total DNA shows that multiple copies of the construct can be inserted in the slime mould genome. In the example reported in Figure 1C, the comparison of the hybridization signals obtained from a stable transformant (st) and the wild-type (wt)

demonstrates the integration of at least 12–15 copies/cell of the cloned region. As suggested by the intense 8 kb band of the *Eco*RI-restricted genomic DNA and by the presence of a unique site for this enzyme in the plasmid used for transformation, insertion occurs predominantly into a single chromosomal site. The excision of the 4.9 kb genomic fragment by *Eco*RI/*Hind*III double digestion, excludes major rearrangements. The small shift to lower molecular weights of the band that contains the endogenous genes, indicates a site of recombination in this region, an event with a relatively low frequency in this type of experiments (see Figure 5B).

Northern analysis of total RNA extracted from the *Dictyostelium* transformant grown in normal oxygen shows the expected proportional increase of the subunit VIIe mRNA, while the alternative transcript remains barely detectable (Figure 1D, O₂). Hypoxia completely reversed the expression pattern (Figure 1D, N₂), as it normally occurs in the parental cells (Sandonà *et al.*, 1995). These results demonstrate that, in the cloned *Bgl*III fragment, both structural genes and the sequences required, in *cis*, for oxygen control are functional. It may also be noticed that under our experimental conditions the presence of the active endogenous genes does not hamper interpretation of the data. The above approach was therefore extended to the investigation of the effects of selected deletions on gene expression (see below).

Figure 2 reports the sequence of the two COX isogenes and their flanking regions. At first sight, the segment shows the typical structure of the *Dictyostelium* DNA, with the two genes embedded in long poly(dA–dT) tracts. Nevertheless, as will be shown later (Figure 5), these 2 kb of genomic DNA contain all the sequence elements needed for oxygen regulation and, ultimately, that are responsible for the presence of COX isoenzymes in the slime mould.

Subunit structure and interactions

The existence of contiguous COX genes in nuclear genomes has never been previously described, but it could be explained by a recent duplication event. This possibility, however, is not supported by the relatively low degree of similarity of the encoded proteins (Figure 3A). Indeed, with 23 invariant residues, subunits VIIe and VIIs are only 44% similar, a value that is the lowest found among COX subunit isoforms, even when the isogenes are dispersed in different chromosomes (Arnaudo *et al.*, 1992). Half of these residues are in the single hydrophobic stretch that, folded in α -helix, spans the membrane (Rizzuto *et al.*, 1991; Tsukihara *et al.*, 1996). As shown in Figure 3B, they cluster on one face of the helix. Since the conservation of the hydrophobic character is the only requirement for a protein surface interacting with lipids, this distribution implicitly indicates their involvement in protein–protein interaction (Figure 3C), suggesting that the two polypeptides compete for the same site in the protein complex. The latter observation may appear obvious, but only if one does not consider the substantial differences that frequently characterize the primary structure of COX subunit isoforms in different organisms (Capaldi, 1990). In this regard, the relatively large sequence divergency of the two *Dictyostelium* polypeptides and the presence of a clearly identifiable element of

Fig. 1. Structural organization of the subunit VII genes of *Dictyostelium* cytochrome c oxidase. (A) Southern blot analysis of subunit VIIe (left) and VIIs (right) genes. The hybridization pattern of total genomic DNA digested with different restriction enzymes is shown. The samples were run on the same 1% agarose gel and transferred to nitrocellulose. The filter was hybridized with a specific DNA probe for subunit VIIe and, after stripping, for VIIs (left and right panel, respectively). Molecular-size markers from *Hind*III-digested phage λ DNA are shown on the left margin. Restriction enzymes are indicated. (B) Map of the region containing the two subunit VII genes, as determined by restriction of genomic DNA and confirmed by sequencing of the cloned 5 kb *Bgl*III fragment. The position of the coding regions is shown by boxes. Three ORFs, present in the DNA segment, are hatched using a motif that suggests their direction of transcription. Horizontal arrows indicate direction (5' $\rightarrow$ 3') and extent of sequencing of fragments obtained by DNA deletion. The map of the two oxidase genes is shown in greater detail in the lower diagram, where open arrows show the direction of transcription. The nucleotide sequence of the segment defined by the two open arrowheads is reported in Figure 2. The restriction enzyme sites are as follows: Bg, *Bgl*III; Sa, *Sau*3A; Ni, *Nsi*I; Ha, *Hae*III; Tq, *Taq*I; EV, *Eco*RV; Ra, *Rsa*I; Hf, *Hinf*I. (C and D) Presence of the oxygen switch in the cloned region. (C) Southern blot analysis of *Eco*RI-digested genomic DNA obtained from the wild-type (wt) and from a *Dictyostelium* stable transformant (st) created by transfection with multiple copies of a plasmid containing the *Bgl*III fragment shown in (B). Total genomic DNA was digested either with *Eco*RI (Ec) or, to excise the cloned 4.9 insert, with both *Eco*RI and *Hind*III (Ec/Hd). The filter was hybridized with random primer-labelled subunit VIIe cDNA. The correspondent expression of the two isogenes under different conditions is shown by Northern blots (D). Total cellular RNA was extracted from exponentially growing amoebae harvested before (O₂) and after a 2 h exposure to a nitrogen environment (N₂). The blots were probed with random primer-labelled subunit VIIe or a subunit VIIs cDNA-specific probe.

Fig. 2. Nucleotide sequence of the subunit VII genes and their flanking regions. The coding regions are indicated by the corresponding amino acids (shown in single-letter code below the sequence). The ORFs with initiation codon at nucleotide 501 and 1521 encode subunit VIIe and subunit VIIs, respectively. Highlighted are restriction sites (dotted underlined), and the nucleotide sequence previously characterized in a subunit VIIe cDNA clone (underlined; Rizzuto *et al.*, 1990). A segment of the intergenic region that, on the basis of the data of this work, plays a key role in oxygen regulation is shown in boldface. This sequence is available from the DDBJ/EMBL/GenBank Data Library under the accession number X99344.

Dictyostelium subunit VII is the only nuclear-encoded subunit of COX that has been characterized in yeast, plant and animal cells. As shown in Figure 4A, in spite of the strictly conserved location of the hydrophobic stretch (horizontal bar), the degree of similarity among the cognate

Fig. 3. Sequence alignment and membrane organization of the two oxidase subunit isoforms. (A) Comparison of the deduced amino acid sequences of the two subunit isoforms. The dash indicates a gap inserted by the computer program (Myers and Miller, 1988) to maintain alignment. Bullets (●) denote identical residues. As shown by the hydropathy plot (Klein *et al.*, 1985), half are localized in the hydrophobic stretch present in the middle of the polypeptide chain. The solid and the dashed lines refer to subunit VIIe and VIIi, respectively. A window of nine amino acids across the length of the protein was employed for calculation. The dotted line divides hydrophobic regions (above) from hydrophilic regions (below). (B and C) Membrane topology. When the hydrophobic segment, that is part of the enzyme membranous sector (see Figure 4), is folded in a α -helix, the invariant residues (darkened area) are clustered on one face of the helix (B), probably in contact with the rest of the protein complex, as indicated schematically in (C).

polypeptides is low. It is also evident, from the dendrogram of Figure 4B, that the differences between the two *Dictyostelium* isoforms are larger than those found for the same subunit in mammals and even comparable with those existing among different organisms. These data provide evidence for the extent of the dramatic change that occurred in the ancestral gene during evolution, to the point that the sequence of the encoded proteins might now be insufficient, without additional information (in this case, the presence and position of the hydrophobic segment), in order to establish homology. These observations suggest that the nuclear-encoded subunits of COX are rapidly evolving, probably to satisfy different function

Fig. 4. Evolution of a nuclear-encoded subunit of COX. (A) Sequence comparison of the two *Dictyostelium* COX isoforms with their homologous counterparts in a yeast and a mammal. Conserved amino acids are boxed. Conservative replacements (period) are marked only for the two alternative *Dictyostelium* subunits. The position of the common hydrophobic stretch is highlighted (horizontal bar). Numbers refer to the subunit VIIIs sequence. (B) Hypothetical phylogenetic tree. The dendrogram, inferred by the method of Higgins and Sharp (1989), condenses and extends the above observations to the different organisms where the same oxidase subunit was characterized (Dd, *D. discoideum*; Sc, *Saccharomyces cerevisiae*; Ib, *Ipomea batatas*; Hs, *Homo sapiens*; Bt, *Bos taurus*; Rn, *Rattus norvegicus*). Subunit nomenclature is according to authors that first identified the polypeptide. The asterisk in one of the two rat genes is to indicate that the protein sequence was deduced from the open reading frame of a putative pseudogene (Suske *et al.*, 1988).

in different organisms, thus increasing the plasticity of this key mitochondrial enzyme. Of particular note is that the existence of isoforms of subunit VII remains a unique feature of the slime mould enzyme, since the presence of multigene families in higher organisms, reported also for this subunit, has been ascribed to processed pseudogenes (Suske *et al.*, 1988).

A short intergenic sequence inversely regulates both genes

As an alternative to a recent gene duplication event, functional reasons could have determined the present structural organization. To investigate this possibility and, more generally, to understand the molecular mechanism of oxygen regulation of gene expression, *Dictyostelium* stable transformants containing multiple copies of different 5' and 3' deleted versions of the sequenced DNA segment (Figure 2) were created and analysed under normal and hypoxic conditions. As shown in Figure 5A, upstream deletions up to 300 bp from the ATG start codon of the subunit VIIe gene have no effect on the regulated synthesis

of both VIIe and VIIs transcripts (rows 1 and 2). The loss of the promoter obviously abolishes transcription of the correspondent mRNA, but still has no influence on the activity of the hypoxic VIIs isogene (row 3).

An unexpected result, however, is the expression under hypoxia of high molecular weight transcripts, with a predominant size of 3 kb, that hybridize with the subunit VIIe probe. Traces of a similar product are also present in stable transformants transfected with the construct shown in row 4 that, in spite of the deletion of the subunit VIIe coding region, still includes 200 bp corresponding to the untranslated trailer region of the mRNA (Figure 2 and Rizzuto *et al.*, 1990). Figure 5B and C analyses the origin of these products. As shown by the Southern blot (Figure 5B), the two transformants contain ~30–40 copies of the selected inserts that, upon excision, exhibit the expected molecular weights. Northern analysis of RNA, extracted from the cells grown under normal and hypoxic conditions, is reported in Figure 5C. Here, the results obtained after hybridization with the double-stranded, random primer-labelled subunit VIIe cDNA probe used

Fig. 5. Deletion analysis. (A) Effect on oxygen-regulated gene expression of progressive upstream and downstream deletions of the genomic DNA fragment shown in Figure 2, monitored by Northern analysis (right). The schematic to the left illustrates the relative length and position of each deletion. The coordinates of the deletion endpoints are given in base pairs 5' of the ATG starting codon of both subunit VIIe (upper number) and VIIs (lower number) genes. For a prompt evaluation of the relative extent of each deletion, a ruler is reported at the top of the figure. Total RNA was extracted from stable transformants containing multiple copies of the deleted region, after vegetative growth either in the presence (+) or in the absence (-) of oxygen. In the latter case, as previously described (Sandonà *et al.*, 1995), cells were exposed to a nitrogen atmosphere for 2 h before harvesting. Under these conditions, the expression of the endogenous genes is negligible. For this reason, in the parental cells (wt) their presence as a single copy is highlighted by a dashed scheme. The comparison of the signals obtained after hybridization of the different sample with either a VIIe- or a VIIs-specific cDNA probe shows that a key *cis*-active sequence element for oxygen regulation is located in the middle of the intergenic region (shaded area). As reported in Figure 1C and D, the relative intensities of the bands in the overexpressing transformants reflect the integrated gene copy number. (B and C) Deletion of the promoter region 5' to the upstream gene activates backwards transcription from the downstream hypoxic promoter. Southern blot analysis (B) of the subunit VIIs gene in the wild-type and two transformants previously shown in rows 3 and 4 of (A). Genomic DNA was digested as reported in Figure 1C, either with *Eco*RI (Ec) or with both *Eco*RI and *Hind*III (Ec/Hd) to allow the precise excision of the cloned insert. Autoradiographs of the Northern blot (C) obtained from the same cells after hybridization with a random primer-labelled subunit VIIe cDNA probe (upper panel) and, after stripping, with a single-stranded, antisense-specific DNA probe (lower panel). The position of the subunit VIIe mRNA is shown by the open arrowhead. The size of the transcripts (kb) is shown on the left margin.

in Figure 5A (upper autoradiograph) and, on the same stripped filter, with a single-stranded, sense DNA probe (lower autoradiograph), are compared. The selective cross-hybridization of the latter antisense-specific probe indicates that the large RNA shown in row 3 is transcribed by a polymerase moving 'backwards' from a downstream hypoxic promoter. With the same highly specific probe, the large transcript of row 4 is undetectable. The concatameric structure of the integrated DNA, suggested by the data of Figure 5B, could explain the 3 kb apparent size of most of the transcripts. This is in fact the length that, moving upstream, separates the hypoxic promoter from the first *Dictyostelium* terminator in the adjacent integrated plasmid. The reproducibility of the result (present in all the 12 independent clones isolated), the absence of similar effects in transformants prepared with the different constructs containing the subunit VIIe gene shown in rows 1, 2 and 7–11 (65 clones analysed in all), and the concentration of the large transcripts (even not considering degradation, 3- to 5-fold above the level of the endogenous messenger), suggest that the above observations are not the consequence of occasional rearrangements.

Returning to the data of Figure 5A, a further 300 bp deletion, between 815 and 510 bp, 5' of the subunit VIIs gene (row 5) does not have obvious effects on its oxygen-regulated expression, but transcription is abolished when the sequence upstream of the coding region is reduced to 260 bp (row 6).

Additional information is obtained by the analysis of progressive 3' deletions of the cloned DNA region (Figure 5A, rows 7–11). As shown by the data of row 7, 100 bp downstream of the hypoxic gene appears to be sufficient for correct transcription termination. Their deletion (row 8) obviously induces the synthesis of larger subunit VIIs RNAs, but the expression remains oxygen-controlled even after the loss of about half of the subunit VIIs gene coding region. A quite different behaviour is shown by the normoxic gene. Although a downstream DNA segment of ~600 bp still allows regulated transcription (row 9), a 100 bp deletion 3' to this region makes expression oxygen-insensitive (row 10). A further shortening does not change the result (row 11). Within the limits of experimental error, no significant differences are found between the level of the subunit VIIe mRNA extracted from transformants containing the constructs of rows 10 and 11, grown in the two extreme conditions considered here. The relative averaged values, obtained from seven independent clones, and after correction for the contribution of the functional endogenous genes, differ in fact by <5%, indicating that regulation is completely lost.

Of note is the fact that the 100 bp genomic region essential for repression of the normoxic gene under low oxygen overlaps the DNA segment whose deletion abolishes expression of the hypoxic gene (Figure 5A, shaded area). Its position coincides with a sequence (highlighted in bold in Figure 2) which is significantly richer in C and G than the neighbour DNA, dominated by long poly(A) and poly(T) stretches typical of untranscribed segments of the *Dictyostelium* genome.

The possibility that certain deletions may induce 'backwards' transcription from the normoxic promoter, as in the case described above for the hypoxic promoter, was also tested by using a DNA probe able to recognize the

1 kb region upstream of the subunit VIIe gene. The absence of cross-hybridization on Northern blots, however, rules out this possibility (data not shown).

Overexpression

In *Dictyostelium* mutants able to constitutively and selectively overexpress subunit VIIe (Figure 5A, rows 10 and 11), one would expect the predominant presence of this polypeptide, even under the hypoxic conditions that normally favour the synthesis of the alternative subunit. If this is the case, analysis of these transformants and their comparison with wild-type cells could offer new insights on the role of COX isoenzymes.

This possibility was investigated first by monitoring the level of mRNA and protein in amoebae growing exponentially either in a normal or in an hypoxic (5% O₂) atmosphere. In these conditions, wild-type cells maintain normal doubling time and the switching between the two COX isoenzymes is complete (Schiavo and Bisson, 1989). As shown by the Northern blots of Figure 6A, in the three *Dictyostelium* transformants used in this experiment (st1–st3), the level of the subunit VIIe mRNA is approximately one order of magnitude above the wild-type (wt). The first of them (st1) integrates multiple copies of the functional region (Figure 5, row 1) and it can therefore overexpress both genes as a function of the oxygen tension (Figure 6A and B). In the remaining two cell lines (st2 and st3) only the endogenous copy of the hypoxic gene is active (Figure 6A and B), since in the integrated constructs it has been deleted to allow constitutive expression of the normoxic gene (Figure 5A, rows 10 and 11).

As expected from previous studies (Sandonà *et al.*, 1995), in a normal atmosphere (21% oxygen) the mRNA of the hypoxic subunits is virtually undetectable in Northern blots (Figure 6B, wt). The hybridization signal remains in fact very low even in the presence of 8–10 copies of the functional genes (Figure 6B, st1). This weak trace, equivalent to ~1% of the amount present under hypoxia, is probably the consequence of the inevitable exposure to limited oxygen of the cells during harvesting, rather than a representation of the real value of mRNA concentration in the growing amoebae. Under these conditions, the obvious result is the exclusive synthesis of subunit VIIe in all the different cell lines considered in Figure 6C (21% O₂, open bars). More surprising is the similar concentration of the same polypeptide in the wild-type and in all the overexpressing mutants, in spite of the large difference of the corresponding mRNA levels. A likely interpretation of this result is a rapid degradation of the translated subunit VIIe, when not immediately engaged in the enzyme assembly.

A parallel analysis of the cells grown under hypoxic conditions adds further information. As reported in Figure 6B, in the transition from 21% to 5% oxygen, the level of the subunit VIIs transcript increases, but only up to 30–40% of the maximum value found after a short exposure to nitrogen (wt, N₂). Conversely, both in the wild-type and in st1, the alternative subunit VIIe mRNA decreases up to 20–30% of its concentration in normal oxygen (Figure 6A). In spite of the comparable levels of the two messengers, however, the hypoxic subunit is the only isoform present in these conditions (Figure 6C, wt and st1, solid bars). Moreover, the concentration of this

Fig. 6. Specific structural features of the two subunit isoforms increase the oxygen sensitivity of the cell response. (A–C) Overexpression. Quantitative Northern (A and B) and Western (C) analysis of mRNA of wild-type (wt) and stable transformants containing multiple copies of the cloned region (st1) or its 3' deleted versions (st2 and st3), able constitutively to overexpress the normoxic subunit. Autoradiographs were overexposed to make the signals of the parental cells visible. Numbers below bands of the Northern blots indicate the relative cellular concentration of the transcripts in a normal (21% O₂) and in a hypoxic (5% O₂) or nitrogen (N₂) environment. The corresponding level of the two oxidase subunit isoforms is shown by the histogram. Data were corrected for unequal loading as described in Materials and methods. (D) Remodelling of the switch. The diagram shows the relative level of subunit *VIIe* (○) and *VIIIs* (●) mRNAs in vegetative *Dictyostelium* amoebae, growing under different oxygen tensions. Each point is the mean of at least four independent experiments. The concentrations of the two proteins (□, ■) are from published work (Bisson and Schiavo, 1986; Schiavo and Bisson, 1989). For a better evaluation of the data, the oxygen concentration in the medium (mM) and the corresponding value in the surrounding atmosphere (% v/v) at equilibrium are reported in the lower and upper abscissa, respectively.

polypeptide in the overexpressing transformant st1 is consistently higher than in the parental cells. Thus, subunit *VIIIs* appears to be more stable than its normoxic counterpart and, even if not assembled, it can survive longer in the mitochondrial membrane.

A possible consequence of this different behaviour is suggested by the data obtained from the transformants st2 and st3 that constitutively overexpress only the subunit *VIIe* gene. Under 5% oxygen, in these mutants the ratio between the normoxic and the hypoxic transcripts is ~20:1 to 30:1 (compare Figure 6A with B). Nevertheless, subunit *VIIIs* is present in more than half of the enzyme molecules (Figure 6C).

These observations can explain the apparent paradox shown by Figure 6D, that quantitatively compares mRNA and protein synthesis in vegetatively growing *Dictyostelium* amoebae as a function of the oxygen tension measured in the culture medium. It may be noticed that the transcriptional switch operates effectively between anoxia and 7–8% oxygen, while the subunit change predominantly occurs in a concentration range between 5% and 15%. The asynchrony of the two events is likely to be the result of the different stability of the two polypeptides that can influence the efficiency of their assembly in the protein complex. The low amount of the hypoxic mRNA transcribed between 10% and 15% oxygen is in fact sufficient to produce enough protein to compete effectively with the normoxic subunit. The net result is a shift of the sensitivity of the response toward conditions of mild hypoxia.

Discussion

Although several genes encoding subunits of COX have been isolated, the molecular mechanisms that coordinate their expression remain largely unknown. Major problems are, in addition to the complex structural organization of the enzyme, its dual genetic origin and the synthesis of alternative subunit isoforms, triggered by environmental or developmental stimuli (Capaldi, 1990). This latter aspect is complicated by the simultaneous involvement of different COX subunits in different organisms and by the dramatic enzyme concentration changes that frequently follow the transition from one oxidase form to another (Hodge *et al.*, 1989; Bonne *et al.*, 1993; Kim *et al.*, 1995; Schagger *et al.*, 1995).

In this general context, the example offered by *Dictyostelium* is relevant for several reasons: (i) a single subunit of the protein complex is involved (Bisson and Schiavo, 1986); (ii) the switching is exclusively induced by oxygen, a substrate of COX (Schiavo and Bisson, 1989); (iii) the process occurs with only minor changes of the cellular enzyme concentration; (iv) the sensitivity is high and the cell response is relatively fast for a eukaryotic system (Sandonà *et al.*, 1995); and (v) the subunit replacement is completed in an oxygen concentration range that appears compatible with the life style of this strictly aerobic organism.

The data presented here now offer a molecular explanation for this precise control. The key feature appears to reside on the structural organization of the genes that encode the two subunit isoforms, in particular on the 100 bp sequence located in the middle of the intergenic

Fig. 7. A model for COX subunit VIIe/VIIi gene switching. In normal oxygen (normoxia), only the *coxE* promoter (open semi-circle) is active. Its presence does not appear to affect *coxS* that remains silent, even after deletion of the upstream gene (see Figure 5). In low oxygen (hypoxia), a *cis*-active sequence element, present in the middle of the intergenic sequence (shaded semi-circle), leads to preferential expression of the hypoxic genes simultaneously inhibiting the upstream promoter. The interaction between the two regulatory regions, mediated by *trans*-acting factors (composite black box) activated by the oxygen-sensing mechanism, is further supported by the loss of directionality of the hypoxic promoter after deletion of 200 bp 5' to the upstream gene. Arrows indicate active mRNA synthesis, while the double arrowhead is to recall the presence of multiple transcripts.

region. As indicated by the data reported in Figure 5, this short DNA segment contains the *cis*-active regulatory element(s) that, in low oxygen, can activate transcription of the hypoxic gene and simultaneously silence the normoxic gene. Its deletion leads in fact to constitutive expression of subunit VIIe. The likely interpretation of these observations is a productive interaction between an oxygen-responsive element(s) present in the intergenic DNA segment and the promoter region of the normoxic gene, mediated by hypoxia-activated transacting factors (Figure 7). The hypothesis is further supported by the 'backward' transcription from the hypoxic promoter, detectable after deletion of 200 bp upstream of the coding region of the normoxic gene (Figure 5D) as a possible result of induced structural changes in the transcription complex (Figure 7, lower diagram). This intimate relationship between separated regulatory sequences explains why the two isogenes have remained physically linked, in spite of the relatively large sequence divergency of the encoded proteins (Figure 3). Their translocation to different chromosomes, a common feature of oxidase isogenes in other eukaryotic organisms (Arnaudo *et al.*, 1992), or simply their segregation in different genomic regions, would in fact result in the complete loss of regulation of the subunit VIIe gene and in a decreased efficiency of subunit VIIi mRNA synthesis in low oxygen.

Among the several cases of adjacent genes that share a common regulatory region, some apparent similarities with the *Dictyostelium* system can be found in the organization of the DNA region responsible for developmental changes in expression of β -globin isotypes in chickens (Choi and Engel, 1988). Again, an intergenic *cis*-active element is essential for the alternative expression of the

tandemly arranged adult β - and embryonic ϵ -globin genes. The transition from ϵ to β , however, occurs by a competition between the two promoters for the intergenic segment that behaves as an enhancer. In this respect, the dual nature of the *Dictyostelium* regulatory sequence, that can serve simultaneously a positive and a negative role, constitutes a substantial difference and represents a novel finding that deserves future investigation.

The presence of the transcriptional switch does not completely explain the high sensitivity to oxygen of the subunit change previously noticed at the protein level (Schiavo and Bisson, 1989). In this connection, the different stability of the two alternative polypeptides, shown by the analysis of the overexpressing mutants, provides additional information. Mitochondria have an intrinsic protein degradation system to avoid accumulation of unassembled subunits or abnormal intermediates (Nakai *et al.*, 1995; Nijtmans *et al.*, 1995; Pearce and Sherman, 1995; Arlt *et al.*, 1996). Its efficiency is shown in *Dictyostelium* by the level of subunit VIIe that remains essentially unchanged even after a 10-fold increase of the corresponding mRNA, induced by the integrative transformation (Figure 5), and in other organisms by the diminution or loss of COX nuclear and mitochondrial subunits after disruption of single selected genes (Capaldi, 1990; Nakai *et al.*, 1995; Pearce and Sherman, 1995). The above observations suggest that the protein degradation system may play a significant physiological role in the biogenesis of the complex. This hypothesis is supported by the behaviour of the hypoxic isoform which, if over-expressed, can, unlike subunit VIIe, increase its concentration above the value dictated by the enzyme stoichiometry. Although protein stability is only one facet of the problem concerning the assembly of a large multisubunit complex it is conceivable that, in the dynamic process involving a competition between the two oxidase isoforms, the more stable subunit VIIi has increased chances of a productive association.

The above observations can account for the efficient synthesis of 'hypoxic' COX molecules that still occurs at close to normal oxygen concentration, in spite of the very low transcription rates of the subunit VIIi gene (Figure 6D). Ultimately, this allows *Dictyostelium* to respond to oxygen changes when it should be most needed for a strictly aerobic microorganism, namely at the beginning of the transition from normoxia to hypoxia, perhaps increasing the probability to either evade a hostile environment or prepare a suitable defence (Sandonà *et al.*, 1995). Oxygen is in fact required for correct development and sporulation, though *Dictyostelium* amoebae inhabit soil, humus and animal dung where this element is certainly limiting (Loomis, 1982; Sandonà *et al.*, 1995). The simple contrast between these two aspects might be at the origin of the ability to sense oxygen evolved by the organism, which is also clearly evident in the sophisticated control of the switch.

In this connection, an obvious question concerns the role of COX in the cell response to hypoxia. As shown by Figure 4, *Dictyostelium* subunit VII corresponds to mammalian subunit VIc. On the basis of the recently published crystal structure of the bovine enzyme (Tsukihara *et al.*, 1996), this polypeptide selectively contacts subunit II, one of the two catalytic subunits of the enzyme.

In particular, its C-terminus interacts with the cytoplasmic domain known to be involved in cytochrome c binding and in the first step of electron transfer from this substrate to Cu_A (Bisson *et al.*, 1982; Babcock and Wikstrom, 1992). If, as suggested by the data of Figure 3, the two subunit isoforms compete for the same site in the protein complex, their reciprocal replacement should considerably modify charge distribution on the catalytic domain of subunit II and eventually perturb its structure. Indeed, of the last 20 residues that constitute the C-terminus, subunit VII_S contributes 12 charged amino acids versus seven provided by subunit VII_E, a situation made even more interesting by the observation that only three of them are invariant. *In vitro* analyses of functional differences between the two alternative forms of the slime mould enzyme have proved to be difficult. In this respect, the cloning of the two isogenes and the elucidation of the basic features of the switching mechanism offer now a variety of *in vivo* approaches that hopefully will shed light on this complex problem.

Materials and methods

Cell culture

Wild-type *Dictyostelium* amoebae (strain AX3) and transformants were grown axenically in HL5 medium (Watts and Ashworth, 1970) at 22°C in suspension as described previously (Bisson and Schiavo, 1986). They were always kept at a concentration of 0.5–5 × 10⁶ cells/ml, i.e. in the exponential phase of growth. Changes of the oxygen tension were produced by fluxing the culture flasks with suitable nitrogen/oxygen mixtures (Schiavo and Bisson, 1989; Sandonà *et al.*, 1995). Further details are reported in the figure legends.

Gene cloning and sequencing

Genomic DNA from strain AX3 was prepared, digested with *Bgl*III and run on preparative agarose gels. Slices corresponding to predicted DNA fragment size of 5 kb were excised and the DNA extracted using the Qiaex kit for nucleic acid extraction (Qiagen GmbH, Dusseldorf, Germany). The presence of the DNA fragment containing the genes in the different fractions was tested by dot blotting. The fragments were then ligated into the *Bam*HI site of a pUC19 vector. From the restriction map of the subunit VII genes, we expected to obtain recombinants containing both genes including the promoter-proximal and -distal halves. The ligation mixture was directly used for bacterial transformation, by electroporation, into strain DH5 on LB/ampicillin plates. Screening of the genomic mini-library was then carried out by hybridization with a cDNA probe of subunit VII_E and a 100 bp genomic fragment of the subunit VII_S gene, labelled by the random-primed method. For confirmation that the desired clone was identified, restriction digestions were carried out on plasmid minipreps. Double-stranded DNA sequencing was carried out by the chain termination method after generation of nested sets of deletions in the cloned genomic fragment (Sanger *et al.*, 1977). Deletions were generated by digestion of clones containing the insert in the two different orientations with unique restriction enzymes present in the polylinker region, using the exonuclease III/mung bean nuclease degradation system of DNA as described (Sambrook *et al.*, 1989). After treatment with Klenow DNA polymerase and religation, the deleted plasmids were used for bacterial transformation. Clones from the deletion library, containing inserts for overlapping sequences, were screened by restriction enzyme mapping.

Transformation of *D.discoideum*

Stable transformants were obtained by electroporation according to Dynes and Firtel (1989). Clones were selected and grown in HL5 medium supplemented with G418 (20 µl) as described previously (Rizzuto *et al.*, 1993).

Other methods and procedures

Mitochondria were isolated and analysed by Western blot as reported (Sandonà *et al.*, 1995). RNA extraction and Northern blot analyses were performed according to published procedures (Nellen *et al.*, 1987;

Sambrook *et al.*, 1989). DNA probes were ³²P-labelled either with random primer labelling kit (Boehringer-Mannheim) or by primer extension. ³²P-labelled coding strand (sense) DNA probes were synthesized using the M13 mp19 system (New England Biolabs) as described (Sambrook *et al.*, 1989). The filters were imaged and quantified using the Packard InstantImager™ electronic autoradiography system. After stripping, filters were rehybridized with *guk*, a cDNA of a mitochondrial enzyme used here as an internal control for RNA loading (Sandonà *et al.*, 1995). Other recombinant DNA techniques were performed according to standard procedures (Sambrook *et al.*, 1989).

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