Structure of the respiratory NADH:ubiquinone oxidoreductase (complex I)

Nikolaus Grigorieff

Three-dimensional structures of NADH:ubiquinone oxidoreductase (or complex I) from the respiratory chain of mitochondria and bacteria have been recently studied by electron microscopy. The low-resolution structures all reveal a characteristic L shape for complex I; however, some of the differences among these structures may have important implications for the location of the functional elements of complex I, for example, the ubiquinone-binding site.

Addresses
WM Keck Institute for Cellular Visualization, Rosenstiel Basic Medical Sciences Research Center, Brandeis University, 415 South Street, Waltham, MA 02454-9110, USA; e-mail: niko@brandeis.edu

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Abbreviations

3D	three-dimensional
EPR	electron paramagnetic resonance
Fe–S	iron–sulfur
FMN	flavin mononucleotide
IP	iron–sulfur protein
NADH	nicotinamide adenine dinucleotide

Introduction

Nicotinamide adenine dinucleotide:ubiquinone oxidoreductase (NADH:ubiquinone oxidoreductase or complex I) is the entry point for electrons into the electron transport chain of mitochondria. It is located in the inner mitochondrial membrane and catalyses electron transfer from NADH to the quinone pool. The intrinsic redox components involve one noncovalently bound flavin mononucleotide (FMN) [1], at least six electron paramagnetic resonance (EPR)-detectable iron–sulfur (Fe–S) clusters [2–4,5•] and at least two distinct protein-bound species of quinone [5•,6–8]. Electron transfer is coupled to proton translocation from the matrix, across the membrane, into the intermembrane space, with 4–5 protons transferred per NADH [9–17]. The proton gradient established by complex I and by the subsequent complexes in the respiratory chain (cytochrome c:ubiquinone oxidoreductase or complex III and cytochrome c oxidase or complex IV) is utilised by ATP synthase to synthesise ATP from ADP and inorganic phosphate.

Complex I has been studied in mammalian cells (reviewed in [18]), plants (reviewed in [19]), fungi (reviewed in [20] for *Neurospora crassa*) and bacteria (reviewed in [21]). Table 1 lists the subunit composition of the *Escherichia coli*, *N. crassa* and bovine enzymes and indicates homologies among subunits. The bovine enzyme has a combined molecular mass of more than 900 kDa [22]. It consists of 43 different subunits, each of which has been characterised, except for a 10 kDa subunit [23] that seems to be part of hydrophobic subcomplex Iβ (see below). Seven of the hydrophobic subunits are encoded in mitochondrial DNA and the remainder are nuclear gene products. The *N. crassa* enzyme has at least 35 different subunits, which can be detected by gel analysis [24]. Their combined molecular mass is about 700 kDa [25,26]. Seven subunits are encoded in mitochondrial DNA and are homologues of the proteins encoded by bovine mitochondrial DNA. Sixteen of the known nuclear-encoded subunits have bovine homologues (Table 1), although some of them have not been sequenced. Complex I from *E. coli* has a combined molecular mass of about 530 kDa and was first determined to have 14 different subunits [27]. Seven are homologous to the mitochondrially encoded subunits. The remaining subunits are homologous to nuclear-encoded subunits of the bovine and *N. crassa* enzymes. Recently, it was found that the *E. coli* genes *nuoC* and *nuoD* are fused and that the protein products form one subunit, NuoCD [28,29]. Genes *nuoC* and *nuoD* are also fused in *Buchnera aphidicola* [30]; however, they are not fused in *Paracoccus denitrificans* [31], *Thermus thermophilus* HB-8 [32] and *Rhodobacter capsulatus* [33], thus encoding 14-subunit enzymes. These 14 conserved subunits form a minimal ensemble for complex I [34].

Substantial progress has been made in the past four years in the understanding of the function of respiratory complexes with the determination of the atomic structures of complex III [35–37] and complex IV [38–42]. To date, however, the atomic structure and detailed reaction mechanism of complex I are unknown. Over the past two years, the three-dimensional (3D) structures of the *N. crassa* [43••,44••], *E. coli* [44••] and bovine enzyme [45••] have been determined to 28 Å, 34 Å and 22 Å resolution, respectively, by electron microscopy. These structures will be reviewed here, together with recent results from studies using NADH oxidoreductase inhibitors to map the structure of the ubiquinone-binding site(s) in eukaryotic enzymes.

The three-dimensional structure of complex I

Bovine complex I can be dissociated into two subcomplexes, known as Iα and Iβ, containing 23 and 17 subunits, respectively [46]. Subcomplex Iα (molecular mass 540 kDa) contains mostly hydrophilic subunits, including those that bind NADH and FMN, and all the Fe–S clusters that have been defined by EPR (reviewed in [5•]). Homologues of seven of its subunits are present in a small form of the *N. crassa* enzyme that is produced by inhibiting mitochondrial protein synthesis with chloramphenicol [47]. For example, both of them contain the 51 kDa FMN-binding subunit and they can both transfer electrons from NADH to quinones. Subcomplex Iα also contains Fe–S cluster N-2, however, whereas the small form of the

N. crassa enzyme does not [18]. It is generally accepted that cluster N-2 participates in the electron transfer reaction of complex I, as the direct donor of electrons to membrane-bound ubiquinone [48,49]. Subcomplex Iα retains a few hydrophobic subunits, but mostly they are hydrophilic, suggesting that most of the subcomplex lies outside the membrane [46].

The first structural information on complex I was obtained from the *N. crassa* enzyme by electron microscopy of two-dimensional crystals and noncrystalline, detergent-solubilised preparations (single particles) of complex I [25,50]. It revealed the characteristic L shape of the enzyme, with one arm of the L embedded in the inner mitochondrial membrane (the hydrophobic membrane domain) and the other arm projecting from the membrane into the mitochondrial matrix (the hydrophilic matrix domain). The membrane domain was shown to be elongated, with a constriction approximately halfway between its two ends. The matrix domain appeared to be more globular, with a thin finger-shaped extension on one side. The two domains were studied separately and their orientation within the intact complex was not determined.

The recent work on the 3D structure of complex I [43••,44••,45••] was carried out with detergent-solubilised protein and single-particle image analysis. Compared with crystallographic techniques, the single-particle approach has the advantage that crystals are not needed and the attainable resolution is not limited by disorder that could be present in a crystal. This technique gives rise to some problems, however, that may be important for the interpretation of the 3D structures. For example, the resolution may be limited by variability among images of individual particles. Artefacts may be produced both by uneven staining of different domains of the protein particle and by flattening of the particle upon drying, as well as by electron-beam-induced changes in the chemical composition of the stain [51,52]. Therefore, to obtain higher resolution, the protein is usually embedded in ice (electron cryomicroscopy [53]), where it is kept hydrated and its structure preserved. With membrane proteins, the presence of detergent in hydrophobic parts of the protein may add to or subtract from the density of the protein, depending on its density relative to that of the ice, thus giving rise to other artefacts.

Figure 1 shows the 3D structures of the *E. coli*, *N. crassa* and bovine enzymes, as published by Guénebaut *et al.* [43••,44••] and Grigorieff [45••]. All the structures display the characteristic L shape initially determined for the fungal complex. The orientation of the *N. crassa* 3D structure with respect to the inner mitochondrial membrane was verified by antibody labelling the 49 kDa subunit [43••], which is part of the iron–sulfur protein (IP) located in the matrix domain. The orientation of the *E. coli* complex was determined by the alignment of its 3D structure with the *N. crassa* structure [44••]. The orientation of the bovine complex was established using three lines of evidence.

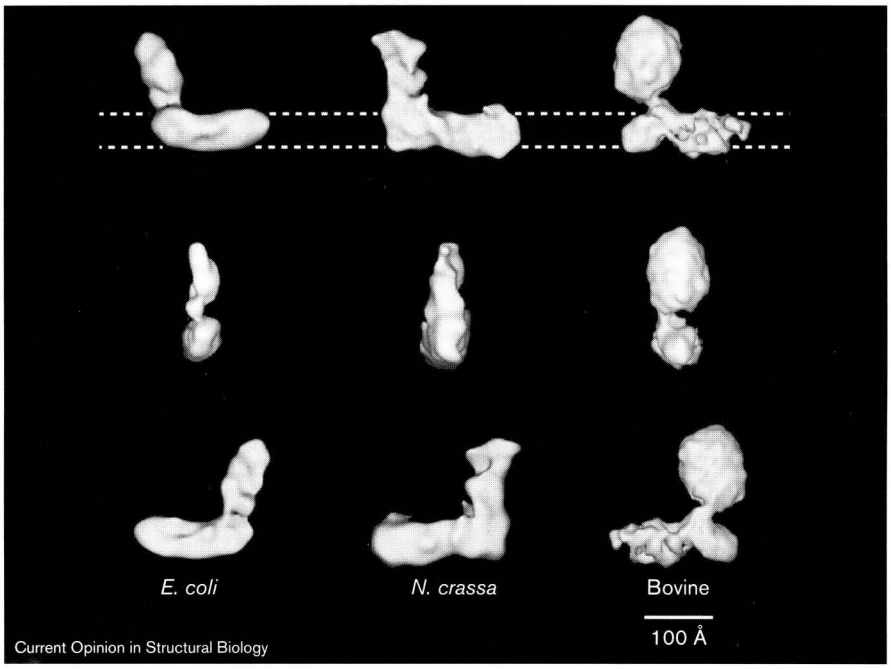
Table 1

Subunits of complex I in the *E. coli*, *N. crassa* and bovine enzymes. Some subunits of complex I from *N. crassa* have not been identified and sequenced. The nuclear-encoded subunits are listed according to their apparent molecular mass, as determined by SDS PAGE, starting with the largest subunit. The location of bovine subunits in subcomplex Iα (mainly hydrophilic) or Iβ (hydrophobic) is indicated in the last column. Eight subunits were not detected in either subcomplex (marked as ND), but are subunits of the intact enzyme. Subunit SDAP is present in both subcomplex Iα and Iβ.

E. coli	*N. crassa*	Bovine	
		Nuclear-encoded subunits	**Location**
INuoG [27]	78 kDa [85]	75 kDa (IP) [86]	Iα
NuoF [27]	51 kDa [85]	51 kDa (FP) [87]	Iα
NuoD* [27]	49 kDa [88]	49 kDa (IP) [89]	Iα
–	?	42 kDa [59]	Iα
–	40 kDa [90]	39 kDa [59]	Iα
NuoC* [27]	30.4 kDa [91]	30 kDa (IP) [92]	Iα
NuoE [27]	24 kDa [93]	24 kDa (FP) [94]	Iα
–	?	B22 [95]	Iβ
NuoI [27]	21.3c kDa [96]	TYKY [97]	Iα
–	12.3 kDa [98]	PDSW [95]	Iβ
NuoB [27]	19.3 kDa [20]	PSST [99]	Iα
–	20.8 kDa [100]	PGIV [101]	Iα
–	?	ASHI [95]	Iβ
–	?	SGDH [95]	Iβ
–	?	B18 [95]	Iβ
–	21 kDa [102]	18 kDa (IP) [95]	Iα
–	?	B17.2 [23]	ND
–	?	B17 [95]	Iβ
–	?	B15 [95]	Iβ
–	14.8 kDa [103]	B14 [95]	Iα
–	?	B14.5a [104]	ND
–	?	B14.5b [104]	ND
–	29.9 kDa [105]	B13 [95]	Iα
–	?	15 kDa (IP) [95]	Iα
–	10.5 kDa [106]	B8 [95]	Iα
–	?	B12 [95]	Iβ
–	?	13 kDa (IP) [95]	Iα
–	?	(10 kDa)†	Iβ
–	ACP [107]	SDAP [108]	Iα and Iβ
–	9.3 kDa [58]	B9 [95]	Iα
–	?	MLRQ [95]	Iα
–	?	10 kDa (FP) [109]	Iα
–	?	AGGG [95]	Iβ
–	?	MWFE [95]	Iα
–	?	MNLL [95]	Iβ
–	?	KFYI [95]	ND
–	21.3a kDa [55]	–	
–	21.3b kDa [57]	–	
–	20.9 kDa [56]	–	
–	17.8? kDa [58]	–	
		Mitochondrially encoded subunits	
NuoH [27]	ND1 [110]	ND1 [111]	ND
NuoN [27]	ND2 [110]	ND2 [111]	Iα
NuoA [27]	ND3 [112]	ND3 [111]	ND
NuoM [27]	ND4 [113]	ND4 [111]	Iβ
NuoK [27]	ND4L [114]	ND4L [111]	ND
NuoL [27]	ND5 [114]	ND5 [111]	Iβ
NuoJ [27]	ND6 [115]	ND6 [111]	ND

*The *E. coli* genes *nuoC* and *nuoD* are fused and proteins NuoC and NuoD form one subunit, NuoCD [28,29]. †This subunit has not been identified, but it seems to be part of hydrophobic subcomplex Iβ [23]. ?, a homologous subunit has not yet been reported; –, a homologous subunit is not present; ND, not detected in subcomplex Iα or Iβ. FP, flavoprotein.

Figure 1



Three-dimensional structures of complex I from *E. coli*, *N. crassa* (as determined in 1998 by Guénebaut *et al.* [44••]) and bovine heart [45••]. Successive views are rotated by 90° about a vertical axis. The position of the lipid bilayer, as determined from two-dimensional and tubular crystals of the *N. crassa* enzyme, is indicated by two broken lines. The structures show the characteristic L shape of complex I, with one arm of the L embedded in the membrane (the membrane domain) and the other arm projecting from the membrane (the matrix domain). The *E. coli* and bovine structures both show a narrowing between their membrane and matrix domains (the stalk) that is not visible in the *N. crassa* complex. The resolutions at which these structures were determined are 34 Å (*E. coli*), 28 Å (*N. crassa*) and 22 Å (bovine). Representations of the *E. coli* and *N. crassa* structures were kindly prepared by Vincent Guénebaut.

First, the two arms of the bovine 3D structure share features with the *N. crassa* complex, as determined by Hofhaus *et al.* [50] in 1991, namely the constriction in the membrane domain and the globular structure of the matrix domain, with its finger-shaped extension. The finger-shaped extension of the arbitrarily oriented matrix domain of the 1991 3D structure of *N. crassa* complex I pointed away from the membrane. In the bovine 3D structure, this extension forms a narrow connection (stalk) between the matrix domain and membrane domain. Second, the orientation of the bovine complex was corroborated by estimates of the molecular masses associated with each domain, based on their fractional volume. Thus, the molecular masses of the membrane domain and the matrix domain (including the stalk) were determined as 370 kDa and 520 kDa, respectively [45••]. The estimated mass of the matrix domain agrees very well with that of subcomplex Iα, which is mainly hydrophilic, but retains some hydrophobicity. The hydrophobicity is assumed to be associated with the stalk, which is in contact with the membrane. A third line of evidence for the orientation of the bovine complex is based on an image of the edge of an ice-embedded tubular crystal of complex I from *N. crassa* [50]. In this image, the matrix domains of a row of complex I units are seen to project from the membrane edge. An average calculated from 70 of these domains clearly shows a narrowing between the matrix domain and the membrane domain that would be consistent with the presence of a stalk. Further evidence for the orientation of the bovine complex relative to the *N. crassa* complex might be obtained from cross-correlation analysis of the two 3D structures; however, this has not been done. The outcome of such an analysis is likely to be strongly influenced by dissimilarities between the two structures that are related mainly to the different embedding media (ice and stain) and differing subunit compositions. Although, in view of the evidence presented, it seems unlikely that the assignment of the membrane and matrix domains in the bovine 3D structure in Figure 1 is incorrect, an unambiguous assignment will require more direct evidence, for example, from immunolabelling of subunits or a higher resolution 3D structure.

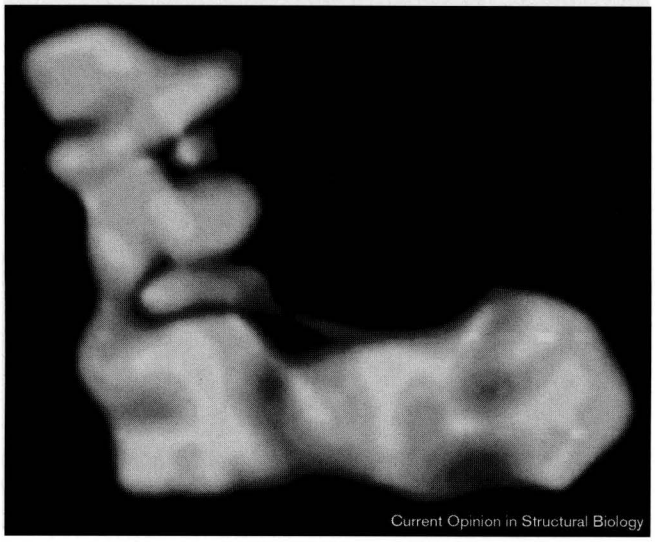
The stalk

The stalk has a diameter of about 30 Å [45••] and is a feature of bovine complex I that is also observed in the *E. coli* enzyme, but not in the *N. crassa* enzyme (Figure 1) [44••]. On the basis of the high degree of homology shared by the subunits of the *N. crassa* and bovine enzymes, however, it seems reasonable to expect an equally high degree of similarity between their 3D structures. The bovine model appears to be in good agreement with the 1991 3D structure of the *N. crassa* enzyme [50], providing that the globular domain in this structure is rotated through 180° about an axis parallel to the membrane. Furthermore, the image of the edge of the tubular crystal of *N. crassa* complex I [50] suggests that the *N. crassa* complex also has a stalk-like feature that connects the matrix domain with the membrane domain. The 3D structure of *N. crassa* complex I presented by Guénebaut *et al.* in 1997 (Figure 2) [43••] shows a large cavity corresponding to a location in the bovine complex that is close to the stalk. This cavity reduces the average density in its vicinity and may have led to the apparent stalk-like feature that is visible in the tubular crystal. The cavity is somewhat hidden inside the matrix domain and was made visible in Figure 2 using a partially transparent structure.

Some of the observed dissimilarities between the bovine and *N. crassa* 3D structures might be the result of a difference between the subunit composition of the two complexes. The *N. crassa* enzyme has four subunits that show no apparent homology with subunits from the bovine enzyme (Table 1). Furthermore, the content of the 42 kDa subunit in the preparation of purified bovine complex I, as analysed by SDS PAGE, appears to be reduced [46,54]. The presence of the four additional subunits in the *N. crassa* enzyme and the (partial) absence of the 42 kDa subunit in the bovine enzyme would account for a total of about 120 kDa molecular mass difference between the 3D structures of these two complexes. The partially hydrophobic character of these subunits [55–59] would be consistent with their location near both the membrane and the stalk. In addition, the observed differences might be related to subunits that have not yet been identified in the *N. crassa* complex. The 200 kDa molecular mass difference between the bovine and *N. crassa* complexes is less likely to explain the dissimilarities in the stalk region, as this would give rise to additional density in the bovine complex, in contrast to the observed structures. Rather, the additional mass of bovine complex I appears to be associated with its globular matrix domain, which is significantly larger than that of the *N. crassa* enzyme (Figure 1). Lastly, as explained earlier, such differences might also be accounted for by stain artefacts. If the stalk is a genuine feature of complex I, then, in the case of the *N. crassa* enzyme, the stain might not have penetrated completely the narrow gap between the matrix domain and the membrane domain. As pointed out in [43••], residual lipid or detergent might appear as additional density in the structure. The invisibility of the stalk in stain preparations would be in agreement with the lower resolution 3D structure of bovine complex I shown by Grigorieff [45••], which was also calculated from images of negatively stained protein and which does not display a stalk either. In the *E. coli* complex, the narrow gap associated with the stalk appears to be absent (Figure 1), making stain exclusion less of a problem. A clearer assessment of possible differences between the bovine and *N. crassa* enzymes will be possible when all of the subunits of the *N. crassa* enzyme have been identified and sequenced.

The stalk is likely to be part of the electron transfer pathway linking the NADH-binding site in the matrix domain with the membrane domain, which is thought to have at least one ubiquinone-binding site. Electron transfer in proteins occurs through either tunnelling over larger distances (up to 20 Å) from one redox centre to the next or movement along covalent bonds (for a review, see [60]). To promote electron transfer in a specific direction and to prevent the capture of electrons by other redox acceptors, which are present in the aqueous and membrane phases, there must be a layer of insulating protein surrounding the electron pathway. It is thought that this layer should be at least 17 to 20 Å thick to be effective [60,61]. With a diameter of 30 Å, the stalk would provide just enough space for insulation. The location of complex I relative to the membrane is only approximately known from Hofhaus *et al.* [50]

Figure 2



Three-dimensional structure of complex I from *N. crassa*, as determined in 1997 by Guénebaut *et al.* [43••]. The structure is resolved at 35 Å and is similar to that determined in 1998 (Figure 1). It shows a large cavity separating the matrix domain from the membrane domain, however, that may be the location of a ubiquinone-binding site. The cavity was made visible using a partially transparent structure [43••]. Kindly prepared by Vincent Guénebaut.

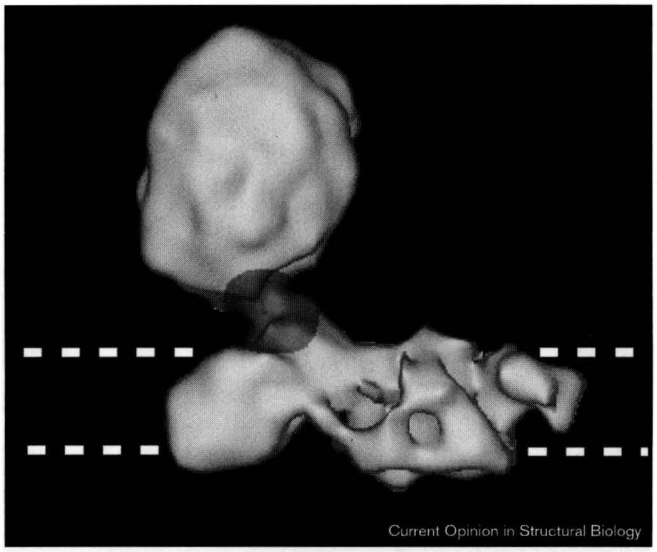
and, therefore, it is also possible that the stalk gains further insulation from the lipid bilayer.

The location of Fe–S cluster N-2 and the structure of a ubiquinone-binding site

The structure and specificity of ubiquinone binding at one site or at multiple sites have been the subject of recent studies [62–68], as well as of earlier studies (reviewed in [69,70]), using a large variety of NADH oxidoreductase inhibitors and ubiquinone analogues. It is now widely accepted that there are at least two ubiquinone-binding sites [7,8,71–74], but their location within the complex remains largely unknown. It has been concluded from spin–spin interaction observed by EPR that the distance between one of the ubiquinone-binding sites and Fe–S cluster N-2 is only 8 to 11 Å [75,76]; however, the interpretation of this observation is disputed by others [77]. The proximity of cluster N-2 and a ubiquinone would be consistent with the transfer of electrons from cluster N-2 to membrane-bound ubiquinone. As cluster N-2 is part of subcomplex Iα [46], which coincides largely with the matrix domain and stalk (see above), it is probable that this cluster is also located close to, if not within, the stalk. The subunit binding cluster N-2 has been suggested to be either TYKY (21.3c kDa in *N. crassa*, NuoI in *E. coli*) [77–79] or PSST (19.3 kDa in *N. crassa*, NuoB in *E. coli*) [76,80,81]; both are protein components of subcomplex Iα, but the precise location of either cluster within subcomplex Iα is unknown.

Enzyme inhibition by a number of bulky substrates [65,66,82], some of which have little structural

Figure 3



Three-dimensional structure of bovine complex I, with the stalk region and sections of the matrix domain and the membrane domain that are close to the stalk shaded in dark grey. Together, they indicate the volume of the complex that may house iron–sulfur cluster N-2 and a ubiquinone-binding site. The position of the lipid bilayer is indicated by two broken lines.

correspondence with ubiquinone or the classical complex I inhibitor rotenone, suggests that the ubiquinone-binding site is fairly nonspecific and located inside a large cavity [66,82]. The cavity may stabilise the intermediate redox product ubisemiquinone [69,83]. If Fe–S cluster N-2 is indeed within the vicinity of the stalk, one might expect such a cavity to be located near the stalk. None of the complexes in Figure 1 is resolved sufficiently to be confident that such a cavity would be visible; however, a promising site may be identified according to the structural information presented so far. This site would be located close to Fe–S cluster N-2, somewhere between the hydrophilic matrix domain and the hydrophobic membrane domain. Based on the bovine 3D structure, this would include the side of the matrix domain facing the membrane domain, the stalk and a section of the membrane domain close to the stalk (Figure 3). This location between the hydrophilic and hydrophobic domains of the complex would be consistent with an amphipathic character of the binding site; however, the narrow gap between the matrix domain and the membrane domain in the bovine 3D structure represents a fairly open cavity whose structure may deviate from that in the *in vivo* complex. Evidence for such a difference between *in vivo* and purified bovine complex I is based on two observations. The first relates to the apparent difference between the 3D structures of the *N. crassa* and bovine enzymes (Figures 1 and 2). As explained earlier, the absence of the stalk in the 3D structure of *N. crassa* complex I may indicate the presence of additional subunits in this area of the complex. The 1997 3D structure of *N. crassa* complex I [43••] clearly reveals a large cavity at the interface between the membrane domain and the matrix domain (Figure 2). A large cavity was also found in the small form of *N. crassa* NADH oxidoreductase [50], which is largely identical to the matrix domain of complex I [47]. This cavity is close to the finger-shaped extension of the subcomplex that may correspond to the stalk in the bovine complex, as discussed earlier. Thus, the bovine 3D structure, as well as the 1991 and 1997/1998 structures of the *N. crassa* complex, is consistent with the idea of a large cavity at the membrane–matrix interface. A second line of evidence for additional subunits in the stalk region of *in vivo* bovine complex I derives from the finding that the ubiquinone reductase activity of the purified complex is partially or completely rotenone insensitive [18,46]. This suggests a major modification of the ubiquinone-binding site in the purified complex that could be related either to the absence of phospholipids or to missing subunits, or both [46,54]. The 42 kDa subunit would be a possible candidate for such a missing subunit, as its reduced content in the purified complex is significant enough to be readily visible by SDS PAGE [46,54].

Recently, the 49 kDa bovine subunit has been implicated in ubiquinone binding [84]. The 49 kDa subunit is part of the matrix domain, but is close to the membrane domain [43••]. Its participation in ubiquinone binding would support the view of a binding pocket that must be in contact with the hydrophobic membrane domain, but is also lined with subunits from the matrix domain. Such a binding pocket would also be consistent with the altered ubiquinone-binding characteristics of subcomplex Iα and the small form of *N. crassa* NADH oxidoreductase. The location of other ubiquinone-binding sites remains unknown.

Conclusions

The low-resolution 3D structures of mitochondrial and bacterial complex I place constraints on the possible locations of functional units of the enzyme. The determination of the precise locations of most of the subunits of complex I has to await further structural studies, for example, using immunolabelling or fractionation into a number of smaller subcomplexes. Nevertheless, some of the visible features at the current level of resolution may be identified as functional elements. Thus, the thin stalk connecting the matrix domain with the membrane domain in the bovine complex appears to be close to Fe–S cluster N-2 and one of the ubiquinone-binding sites. Clearly, a higher resolution structure would shed light on many of the currently investigated topics, for example, the structure, location and number of ubiquinone-binding sites and Fe–S clusters. Higher resolution may be obtained from crystallographic studies, for example, using a water-soluble subcomplex [29].

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- •• of outstanding interest

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