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Review

Cytochrome *bd* terminal oxidase [1]

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Contents

Abbreviations: Aurachin D, 2-methyl-4(^{1}H)-quinolone; DTT, dithiothreitol; FTIR, Fourier transform infrared; HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; $K_{m(O2)}$, Michaelis constant for oxygen; Q/QH_2,quinone/quinol; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole

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[1] All amino acid numbering refers to the *E. coli* enzyme.

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1. General introduction

Most respiratory oxidases are structurally and functionally closely related members of a large group of proton-pumping haem-copper oxidases which also includes mitochondrial cytochrome *c* oxidase [1,2]. The cytochrome *bd* complex is the only well characterised bacterial terminal oxidase which is unrelated to this haem-copper oxidase superfamily. The enzyme functions as a quinol oxidase. Spectral evidence as well as immunological investigations have shown cytochrome *bd* to be widely distributed, particularly in Gram-negative heterotrophs [3]. Spectral features tentatively assigned to cytochrome *d* have also been found in a number of *Bacillus* species [4–9] and some archaebacteria (see [10]), although the function of cytochrome *d* as terminal oxidase has not been demonstrated in all of these organisms. Purifications are reported from various sources, including *Escherichia coli* [11,12], *Klebsiella pneumoniae* [13], *Photobacterium phosphoreum* [14], *Azotobacter vinelandii* [15,16] and various *Bacillus* species [8,9].

This review summarises what we know about structure and function of the cytochrome *bd* complex and indicates unresolved problems which may be belied by the apparent simplicity of the enzyme. The *E. coli* and *A. vinelandii* oxidases are most extensively characterised and show close similarity in their spectral, genetic, immunological and kinetic properties. Therefore, the following paragraphs will concentrate on the cytochrome *bd* complex from these two organisms.

2. Genetics and molecular biology

Four gene loci, *cydA*, *cydB*, *cydC* and *cydD*, involved in the expression of the cytochrome *bd* complex have been identified in *E. coli*. *CydA* and *cydB* code for the two subunits of the oxidase [17]. The genes are contiguous, mapping near minute 16.6 in the *E. coli* chromosome [18]. They are cotranscribed during cytochrome *bd* biosynthesis. *CydA* and *cydB* have been cloned [19] and sequenced [20]. A *cydAB* sequence was also identified in the genome of *A. vinelandii* [21]. Sequence analysis [22] revealed a high degree of similarlity in the derived amino acid sequences from *E. coli* and *A. vinelandii*. In particular, three putative haem ligands (His^{186}, Met^{393}, His^{19}) and a region forming part of the proposed quinol binding site are conserved (Fig. 1). In *E. coli*, a further operon coding for a putative *bd*-type oxidase, *appBC*, has been discovered [23]. Subsequent purification of the *AppBC* protein has confirmed that the enzyme has quinol oxidase activity, with spectral characteristics very similar to the *cydAB*-encoded oxidase [24]. The deduced amino acid sequence of *appBC* shows about 70% similarity to the *cydAB*-encoded subunits of cytochrome *bd* [23], although no cross-reaction with antibodies directed against 'conventional' *E. coli* cytochrome *bd* has been detected [24]. Sequences predicted to encode *bd*-type oxidases have also been found in *Haemophilus influenzae* [25] and *Bacillus subtilis* [26] (Fig. 1).

CydC and *cydD* were also found to be contiguous and mapped near minute 19.2–19.3 on the *E. coli* chromosome [27,28]. Since the gene products are not part of the functional oxidase they may be necessary for haem biosynthesis, transport or assembly. Bebbington and Williams [29] found that *cydD* mutants still produce subunit I and synthesise the haem D containing catalase HPII. This led the authors to conclude that *cydD* was not involved in regulation, oxidase assembly or haem D synthesis, although no data on assembly of subunit II were given. Cloning and sequence analysis [30] has revealed homology with ABC membrane transporters. Poole et al. [30] propose that the function of the *cydC* and *cydD* gene

products is the export of haem into the periplasm. It appears that this function is not specific to haem D or haem components of the *bd* complex, as defects in *cydCD* also affect assembly of periplasmatic *c*-type cytochromes in *E. coli* [31]. However, Goldman and co-workers [32] have found that *cydC* mutants are not only defective with regard to levels of periplasmic haem proteins (apo- and holoprotein) which can not be corrected by addition of haemin, but also have an altered periplasmic redox environment as judged from the reduction level of sulphydryl groups. The authors suggest that the *cydCD* transporter protein may affect some post-secretory step in the assembly pathway of secreted proteins and extracellular domains of membrane-bound enzymes, possibly by some function of *cydCD* in maintaining the periplasmic redox balance.

Gene clusters for oxidases commonly contain genes coding for the protein subunits as well as genes required for haem biosynthesis. For example, the farnesyltransferase converting haem B into haem O is, in *E. coli*, coded by the *cyoE* gene in the gene cluster for the *bo*-type oxidase [33]. However, little is known about the biosynthesis of the chlorin haem D, i.e., a porphyrin with one pyrrole ring saturated [34]. It has been shown that haem B is a precursor of haem D [35], but the reaction may not be enzyme-catalysed. For catalase HPII from *E. coli*, cytochrome *d* appears to be converted in situ from the initially inserted *b*-type haem, using one of the first peroxide substrate molecules [36], and the protohaem in metmyoglobin can be altered to a chlorin by in vitro treatment with hydrogen peroxide [37]. A similar mechanism might operate in the cytochrome *bd* complex.

It has been suggested that in *E. coli* and other enteric bacteria, cytochrome *bd* serves as a high-affinity oxidase to support energy-requiring processes under microaerobiosis and to protect anaerobic processes from inhibition by oxygen [38]. In *E. coli* loss of cytochrome *bd* leads to a lowered ability of the organism to cope with oxidative stress, resulting in, e.g., increased sensitivity to higher temperatures or peroxide [39]. An oxygen scavenging role is also exerted in respiratory protection [40,41] of the oxygen-labile nitrogenase during aerotolerant nitrogen-fixation in *Azorhizobium caulinodans* [42] or *A. vinelandii* [21], an organism with the most active respiratory chain known where cytochrome *bd* is thought to be the major pathway from ubiquinol to oxygen ($\geq$ 70% [43]). The synthesis of cytochrome *bd* is regulated by many factors, the most prevalent being the oxygen content of the medium (for a review, see [44]). Whereas in *A. vinelandii* high aeration of the culture induces an increase in the cytochrome *bd* level [45,46], the second oxidase of this organism, a poorly characterised *b*- or *o*-type oxidase [47–52], dominates under oxygen limiting conditions. It is noteworthy that this regulation is opposite to the *E. coli* system where cytochrome *d* is induced during oxygen limited growth and the coexisting cytochrome *bo* complex prevails at higher oxygen concentrations [53]. The modulation of cytochrome *bd* and *bo* levels in response to oxygen in *E. coli* has recently been linked to the *Fnr* and *ArcAB* regulatory proteins [54–58], two broadly acting regulators responsible, e.g., for the transition between aerobic and anaerobic respiration [59]. Interestingly, the variation of cytochrome *d* levels in response to oxygen (about 2–3-fold increase at oxygen limitation) has been found to be much lower than that of cytochrome *bo* whose expression may increase up to 160-fold on transition to aerobic conditions [54,56]. The *Fnr* and *ArcA* proteins are also involved in transcriptional control of cytochrome *bd* in response to carbon availability where they interact with a further global regulatory protein, the catabolite repressor activator *Cra* [60]. In *A. vinelandii*, transcription of cytochrome *bd* is up-regulated approximately three-fold when nitrogen-fixation is induced. Since the *cyd* genes in this organism differ from the genes for the nitrogen-fixing apparatus (*nif*) in not having binding sites for a *nif*-specific transcription factor (*nifA*) and σ-factor (*ntrA*), *cydAB*, unlike the *nif* genes, is constitutively transcribed even under non-nitrogen fixing conditions. However, the *ntrA* protein or other *ntrA*-dependent gene(s) are required for up-regulation of cytochrome *bd* during nitrogen-fixation [61]. Increased expression of cytochrome *bd* in *A. vinelandii* at elevated oxygen levels is also observed in the absence of diazotrophy. *CydR*, a regulatory gene upstream of *cydAB*, may be involved in this control mechanism since mutations in *cydR* lead to overproduction of cytochrome *bd* and reverse response to oxygen [62].

The *appBC*-encoded, second *bd*-type oxidase in

SUBUNIT I

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                                        ↓ TMH I
CydA E.coli     ML--DIVELSRLQFALTAMYHFLFVPLTLGMAFLLAIMETVYVLSGKQIYKDMTKFWGKLFGINFAL  65
CydA A.vine.    MISESVVDLSRLQFAMTALYHFLFVPLTLGMTFLLAIMESVYVMTGKQVYKDMVKFWGKLFGINFAL  67
AppC E.coli     MW--DVIDLSRWQFALTALYHFLFVPLTLGLIFLLAIMETIYVVTGKTIYRDMTRFWGKLFGINFAL  65
CyoA H.infl.    ML--DVVDLSRLQFALTALYHFIFVPLTLGLSFILVIMETIYVATGKEVYKDMTKFWGKLFGINFAL  65
CydA B.subt.    M---SELVLARIQFASTTLFHFLFVPMSIGLVFMVALMETLYLVKKNELYLKMAKFWGHLFLINFAV  64

                                                TMH II                    
CydA E.coli     GVATGLTMEFQFGTNWSYYSHYVGDIFGAPLAIEGLMAFFLESTFVGLFFFGWDRLGKVQHMCVTWL 132
CydA A.vine.    GVTTGITMEFQFGTNWAYYSHYVGDIFGAPLAIEGLTAFFLESTFIGMFFFGWDRLSKIQHLAVTWL 134
AppC E.coli     GVATGLTMEFQFGTNWSFYSNYVGDIFGAPLAMEALMAFFLESTFVGLFFFGWQRLNKYQHLLVTWL 132
CyoA H.infl.    GVTTGIIMEFQFGTNWSYYSHYVGDIFGAPLAIEALLAFFLESTFVGLFFFGWDRLSKGKHLLATYC 132
CydA B.subt.    GVVTGILQEFQFGLNWSDYSRFVGDVFGAPLAIEALLAFFMESIFIGLWIFGWDRLPKKIHALCIWL 131

                TMH III                                 ↓ TMH IV
CydA E.coli     VALGSNLSALWILVANGWMQNPIASDFNFETMRMEMVSFSELVLNPVAQVKFVHTVASGYVTGAMFI 199
CydA A.vine.    VALGSNLSALWILVANGWMQHPVGAEFNFETMRMELVDFGALLLNPVAQVKFVHTVASGYVTGAVFV 201
AppC E.coli     VAFGSNLSALWILNANGWMQYPTGAHFDIDTLRMEMTSFSELVFNPVSQVKFVHTVMAGYVTGAMFI 199
CyoA H.infl.    VAFGSNLSAMWILVANGWMQAPTGSEFNFETVRMEMTNFLDLWLNPVAQSKFLHTLSAGYVTGAFFV 199
CydA B.subt.    VSFGTIMSSFWILTANSFMQEPVG--FTIKNGRAEMNDFGALITNPQLWVEFPHVIFGALATGAFFI 196

                        TMH V                   Q-domain
CydA E.coli     LGISAWYMLKGRDLAFAKRSFAIAASFGMAAVLSVIVLGDESGYEMGDVQKTKLAAIEAEWETQPAP 266
CydA A.vine.    LAISSYYLLKKRDLGFARRSFAIASAFGMASILSVIVLGDESGYEVGEVQKAKLAAIEAEWETHPAP 268
AppC E.coli     MAISAWYLLRGRERNVALRSFAIGSVFGTLAIIGTLQLGDSSAYEVAQVQPVKLAAMEGEWQTEPAP 266
CyoA H.infl.    LAISSYFLLKGRDFEFAKRSFSVAATFGFIASISVLILGDESGYDIGKAQPVKLAAMEAEFETHPAP 266
CydA B.subt.    AGVSAFKLLKKKEVPFFKQSFKLAMIVGLCAGLGVGLSGHMQAEHLMESQPMKMAASEGLWEDSGDP 263

CydA E.coli     AAFTLFGIPDQEEETNKFAIQIPYALGIIATRSVDTPVIGLKELMVQHEERIRNGMKAYSLLEQLRS 333
CydA A.vine.    ASFTLIGFPNEEEQRTDFAVKIPWVLGIIATRSLDEQVIGIKDLIADHEARIRNGMVRYGLLEELRA 335
AppC E.coli     APFHVVAWPEQDQERNAFALKIPALLGILATHSLDKPVPGLKNLMAETYPRLQRGRMAWLLMQEISQ 333
CyoA H.infl.    APFLPVAIPNTAEMKNDFAIEIPYLGGVIATRSIDKEIIGLKDLQALNETRVRSGIRAYELFTQLRA 333
CydA B.subt.    AAWTAFATIDTKNEKSSNEIKVPYALSYLAYQKFSGSVKGMKTLQAEYE------------------ 312

                                                                  ↓
CydA E.coli     GST-----DQAVRDQFNSMKKDLGYGLLLKRYTPNVADATEAQIQQATKDSIPRVAPLYFAFRIMVA 395
CydA A.vine.    GNK-----SPEKIAAFNEVKDDLGYGLLLKKYTPNVVDASEEQIKQAAKDTIPSVASMFWSFRAMVG 397
AppC E.coli     GNR-----EPHVLQAFRGLEGDLGYGMLLSRYAPDMNHVTAAQYQAAMRGAIPQVAPVFWSFRIMVG 395
CyoA H.infl.    EKKANGQVNEETKAQFNEVKKDLGFGLLLKRYTNNVVDATEEQIKQAARDTIPNVGPNFWAFRAMIA 400
CydA B.subt.    ----------------------------KIYGKG--------------DYIPPVKTTFWSFRIMVG 336

                TMH VI
CydA E.coli     CGFLLLAIIALSFWSVIRNRIGEKKWLLRAALYGIPLPWIAVEAGWFVAEYGRQPWAIGEVLPTAVA 462
CydA A.vine.    AGFAMLILFVCAFWASARKNEESKPWLLKFALYSLPLPWIATQTGWFVAEHGRQPWTIGGVLPTHLS 464
AppC E.coli     CGSLLLLVMLIALVQTLRGKIDQHRWVLKMALWSLPLPWIAIEAGWFMTEFGRQPWAIQDILPTYSA 462
CyoA H.infl.    AGGLIALLTFGAFVQNLRNKVTQIPLLLKVLLWGLPLPWIAIECGWFLAEYGRQPWATYEILPVGVS 467
CydA B.subt.    AGVVMILAALGGLWLNRRKKLENSKWYLRIMIALISFPFLANSAGWIMTEIGRQPWTVMGLMTTAQS 403

                        TMH VIII
CydA E.coli     -NSSLTAGDLIFSMVLICGLYTLFLVAELFLMFKFARLGPSSLKTGRYHFEQ---------SST-TT 518
CydA A.vine.    -ASSLSTGDLWGSLIALIAFYTLLLVVEMYLMIRFARLGPSSLHTGRYHFEQLEQHAVKHASPS-QA 529
AppC E.coli     -HSALTTGQLAFSLIMIVGLYTLFLIAEVYLMQKYARLGPSAMQS-----EQPTQQ----------- 512
CyoA H.infl.    -ASNLSTGDLWFSIGLICALYLAFIIVEMYLMFKYARLGPSALKTGKYYFEQSAK            521
CydA B.subt.    VSPNVTAGSLLFSIIAFGVMYMILGALLVFLFIREIKKGA----------EHDNHHDVPVSTDPFSQ 460


CydA E.coli     QPAR                                                               522
CydA A.vine.    DPQQPVNA                                                           537
AppC E.coli     ------QG                                                           514
CyoA H.infl.
CydA B.subt.    EVYHGISS                                                           468
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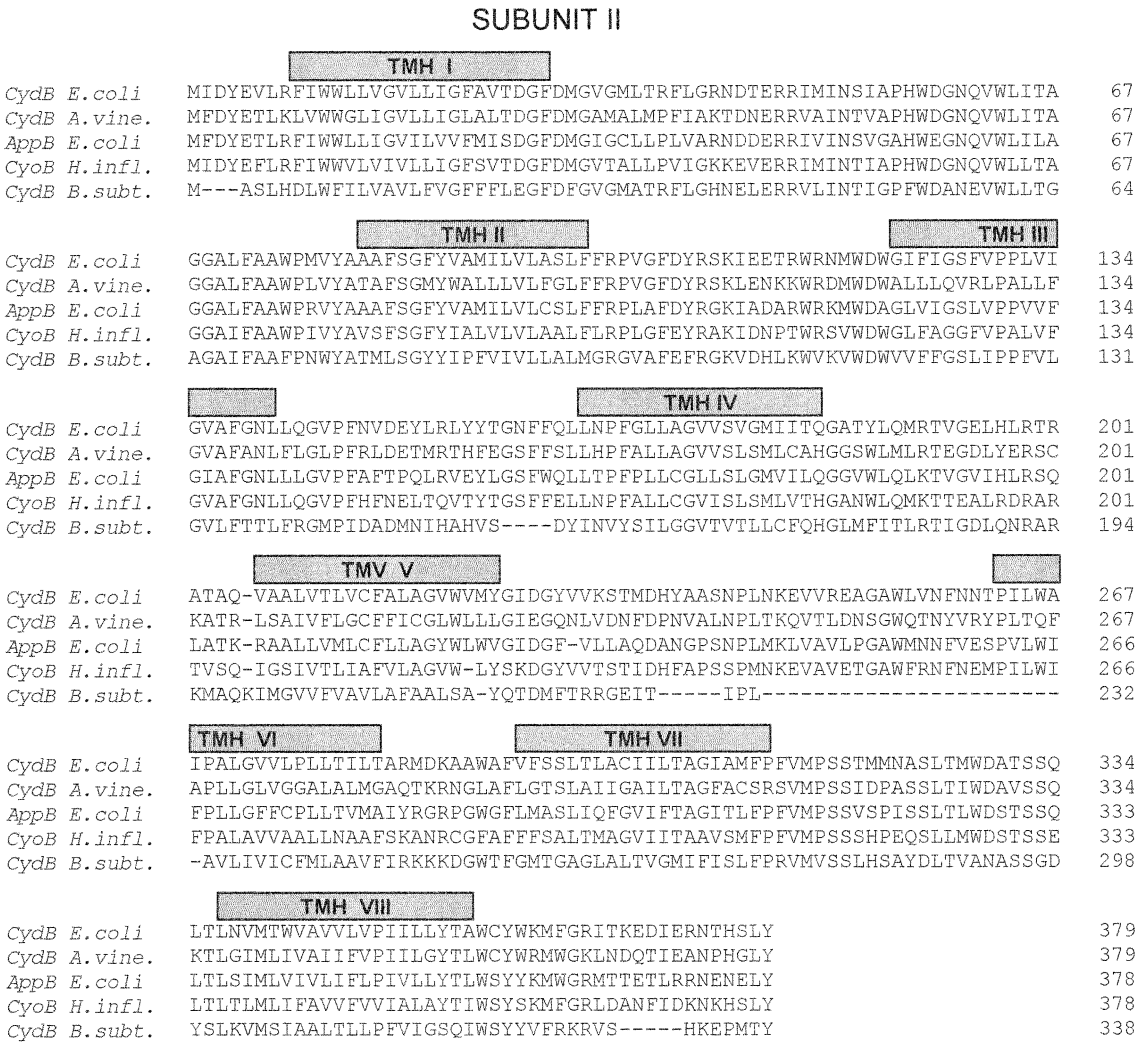

Fig. 1. Alignment of the amino acid sequences of the *A. vinelandii*, *E. coli* and *B. subtilis cydAB*, *H. influenzae cyoAB* and *E. coli appBC* proteins. The amino acid sequences are as deduced from the nucleotide sequences [20,22,23,25,26]. Transmembrane-spanning segments (TMH), His^{19}, the ligands of haem *b*-558 (His^{186} and Met^{393}) and the putative quinol domain are indicated, based on *E. coli cydAB*.

E. coli also appears to be oxygen-regulated with maximal expression under anoxic or microaerophilc conditions [63]. The protein does seem to have physiological significance since *cyo cyd appB* triple mutants show increased oxygen sensitivity compared to *cyo cyd* double mutants [23]. It has not been investigated whether in cytochrome *bd* preparations from *E. coli* contamination of *cyd*-derived oxidase with *AppBC* protein does occur, leading to a possible heterogeneity problem.

3. Oxidase activity and chemiosmotic function

3.1. Enzyme kinetics

Besides the physiological substrate ubiquinol-8, in vitro substrates of cytochrome *bd* include shorter-chained ubiquinols, menadiol, duroquinol and the artificial electron donors like TMPD [11,12]. Oxidase activity depends strongly on the detergent present. Cytochrome *bd* from *E. coli* is inactive in, e.g.,

cholate or octylglucoside, but retains activity in Tween 20 and Triton X-100 [64]. The ubiquinol-1 oxidase activity of cytochrome *bd* does not have a clear pH maximum, but rises from pH 5.5 to reach a plateau around pH ≥ 7.5 [64].

Activities with the different substrates vary widely, in the case of quinols partly because of the steric requirements of different substituents on the quinol ring [65], partly because of varying solubility and partition properties [66]. Solubility limitations may also be the origin of the apparent substrate (quinol) inhibition observed with a number of ubiquinol analogues by Sakamoto and co-workers [65]. Using ubiquinol-1 (plus 2 mM DTT, at pH 7.9) turnover numbers of around 500–1000 $O_2 \cdot s^{-1}$ have been observed with the *E. coli* and *A. vinelandii* oxidases [67]. In contrast, duroquinol is a poor substrate, with a turnover of around 50–100 $O_2 \cdot s^{-1}$ at 1mM duroquinol and a K_m value above 1 mM (for *A. vinelandii* enzyme, Jünemann and Rich, unpublished data), at least one order of magnitude higher than the value for ubiquinol-1 (see below). Similarly, the turnover with TMPD (plus ascorbate) is comparatively low; the V_{max} reaches around 10–30% of that with ubiquinol-1 (plus DTT), with a K_m value in the mM region ([11,12], Jünemann and Wrigglesworth, unpublished data).

Several groups have measured the operational oxygen affinity of cytochrome *bd* with considerable variation of the resulting $K_{m(O2)}$ values, both between different species and for oxidase from the same organism (see [67] and references therein). However, the data led to the thought that the apparent oxygen affinity of cytochrome *bd* from *A. vinelandii*, as indicated by its $K_{m(O2)}$ is much lower (up to 3 orders of magnitude) compared to the *E. coli* enzyme. Most of these measurements were 'one-point' experiments at one particular concentration of reducing substrate. However, the oxidase catalyses a multi-substrate reaction where the observed $K_{m(O2)}$ is modulated by the electron flow through the enzyme. The observed $K_{m(O2)}$ at any particular level of turnover will therefore depend firstly on the concentration of the second substrate, ubiquinol, and secondly on the K_m of the enzyme for ubiquinol. Taking this into account, a comparative study of the *E. coli* and *A. vinelandii* enzymes showed that the limiting $K_{m(O2)}$ values, i.e., extrapolated to saturating ubiquinol concentrations (or maximum turnover), are similar for both oxidases, 2 μM and 4 μM, respectively (at pH 7.9 with ubiquinol-1 plus DTT as electron donor, [67]), in good agreement with a previous report [15]. A larger difference was observed in the K_m for ubiquinol-1 at 42 μM and 220 μM, respectively. The K_m for ubiquinol is sensitive to detergents and lipids, giving rise to variations of up to one order of magnitude between different experimental conditions [11,12,15,64,67]. Although the limiting K_m values for ubiquinol and oxygen are useful parameters to compare the *bd*-type oxidases from various sources, they may not be the values observed under physiological conditions since all enzyme kinetic parameters, turnover and K_m for both substrates, are modulated by the concentrations of both substrates. Such a process would contribute to the regulation of overall oxygen consumption rates as well as flux control between the alternative oxidases in branched respiratory chains.

Kinetic measurements with purified and membrane-bound cytochrome *bd* from *E. coli* and *A. vinelandii* using malate, NADH (with membranes only) or ubiquinol-1 plus DTT as electron donors did not show any substrate (oxygen) inhibition [67], contrary to reports by Poole and co-workers [68,69].

3.2. Chemiosmotic function

Ubiquinol oxidase activity of the purified cytochrome *bd* complex from *E. coli* is accompanied by generation of a transmembrane potential of about 180 mV, negative inside, when reconsituted into proteoliposomes [12,70]. A stoichiometry of 1 H^+/e^- [71,72] can be accounted for by transmembrane movement of electrons combined with formation of a proton gradient by substrate protons, i.e., the release of two protons per quinol molecule oxidised on the periplasmatic side, and uptake of four protons per O_2 molecule reduced on the cytoplasmic side of the membrane, without invoking a proton pump.

Recently, Skulachev's group discovered a Na^+-motive terminal oxidase in *E. coli* [73] which, in further experiments with mutant strains lacking either cytochrome *d* or cytochrome *o*, has been correlated with the *bd* complex [74]. Consistent with this hypothesis both sodium-motive respiration and cytochrome *d* can be induced in *E. coli* under condi-

tions of high Na^+, low $\Delta\mu_{H+}$, e.g., in the presence of protonophorous uncouplers or at alkaline pH, or low (μM) concentrations of cyanide which inhibit cytochrome *bo* but not *bd* [74–76]. However, in Bacillus FTU, Na^+-motive respiration does not appear to be related to cytochrome *d* [6]. It is difficult to see how a mechanism based on substrate protons can be involved for Na^+-motive systems, and the role of cytochrome *bd* as sodium-motive oxidase is still controversial.

3.3. Inhibitors

A number of inhibitors has been reported for the *bd*-type oxidase. These fall into two classes, haem ligands such as cyanide acting at the oxygen binding site and compounds known to be quinol antagonists in other systems. Molecules binding to the oxygen reaction site tend to be comparatively weak inhibitors. Most notably, several authors have pointed out the relatively high resistance of cytochrome *bd* to cyanide, with estimations of the K_i value in the 10^1–10^2 μM region [43,77,78] — the corresponding value for bovine cytochrome oxidase is 50–100 nM [79,80]. Low sensitivity with mM amounts necessary for inhibition is also observed with nitrite [81] and azide [11,12,82]. This low inhibitory potency of anionic haem ligands may reflect a hydrophobic environment at the oxygen binding site. However, in the case of ligands to ferric haem *d* such as cyanide and azide K_i values may be overestimated since only a small fraction of the enzyme population can react with the inhibitor (see below). Although carbon monoxide does bind to ferrous haem *d* with high affinity, as discussed below, a substantial residual activity ($>20\%$) is still observed at 0.5–1 mM CO [43,82]. This may reflect the use of haem *b*-595 as an alternative oxygen binding site [69,83,84], but is probably due to a comparatively high rate constant for haem *d*-CO dissociation ([85], also see Table 3).

Most of the Q-site inhibitors of cytochrome *bd* have inhibitor constants in the 10^1–10^2 μM range, e.g., HQNO, antimycin A, UHDBT, piericidin A [86,87]. However, the quinolone-type compounds aurachin C and D and their alkyl-substituted derivatives were found to be extremely potent, close to stoichiometric inhibitors of the oxidase [87,88]. Aurachin D and its derivatives are of particular interest since, in *E. coli*, these compounds, unlike the aurachins C, are specific for cytochrome *bd* and do not inhibit cytochrome *bo*.

The Q-site antogonists have not been systematically screened for their effect on the TMPD oxidase activity. However, those tested (antimycin A, HQNO, UHDBT, decyl-aurachin D) did not inhibit TMPD oxidation [86,88].

4. Subunit composition and redox centres

The purified cytochrome *bd* complex from *E. coli* contains two subunits with molecular weights of 58 kDa (subunit I) and 43 kDa (subunit II) as determined by SDS-gel electrophoresis [11], consistent with the values of 48 and 36 kDa calculated from the amino acid sequence as deduced from the DNA sequence [20]. Similar properties have been reported for the purified *A. vinelandii* oxidase (52 kDa and 36 kDa, calculated from Fig. 1 in [15]; 50 kDa and 31 kDa [16]; 50.1 kDa and 35 kDa, calculated from sequence data in [22]),the *appBC*-encoded enzyme from *E. coli* (43 kDa and 27 kDa [24]) and preparations of cytochrome *bd* from *Klebsiella pneumoniae* (52 kDa and 35 kDa [13]), *Photobacterium phosphoreum* (54 kDa and 41 kDa [14]), *B. stearothermophilus* (52 kDa and 40 kDa [8]) and *B. firmus* OF4 (35.3 kDa and 23.5 kDa [9]). (It should be noted that the values of 57.4 and 42.5 kDa for *E.coli* [20] and of 59.7 and 42 kDa for *A. vinelandii* [22] quoted in the literature for the calculation from sequence data are higher because no allowance for water removal on peptide bond formation was made.) Antibodies raised against the subunits of the *E. coli* complex have been used to show that proteins related to both subunits can be found in Gram-negative organisms where cytochrome *d* is detected optically [3]. Cross-reactivity has also been reported for *Bacillus firmus* OF4 [5]. In the native form the enzyme probably functions as a heterodimer [89]. Some tightly bound lipopolysaccharide has been detected immunologically [90].

Cytochrome *bd* contains three types of haem redox centres, haem *d* and one molecule each of haems *b*-558 and *b*-595, but no non-haem iron or copper [11,12]. Although some data have been interpreted as being compatible with the presence of two *d*-type

haems per complex [91,92], a stoichiometry of one haem *d* has been established by quantitative EPR measurements, ligand binding, determination of the iron and haem contents and single turnover experiments [16,81,85,93–95]. Unlike cytochrome *bo* which contains a tightly bound ubiquinone [96] no quinone has been detected in purified preparations of cytochrome *bd* from *E. coli* [11,12].

The two *b*-type cytochromes in the complex have distinct spectral and electrochemical properties. One of them, *b*-558, is low-spin, hexacoordinate, whereas *b*-595 is a high-spin, pentacoordinate haem. Spectral analysis also indicates that haem *d* is directly involved in oxygen binding. Construction of *cydA* and *cydB* mutants located haem *b*-558 on subunit I and demonstrated that subunit II is necessary for binding of haems *b*-595 and *d* [17]. Subunit I can be expressed independently in *cydB* defective mutant strains, facilitating purification of the cytochrome *b*-558 component [97].

5. Spectral and electrochemical properties

5.1. Optical

In the visible region, the spectrum of cytochrome *bd* 'as prepared' is dominated by an intense absorbance peak near 650 nm due to a stable 'oxygenated' compound, with a shoulder at 670–680 nm arising from the oxo-ferryl form of haem *d* [95,98,99]. In the reduced form, the haem *d* α-peak shifts to about 630 nm, whereas ferric haem *d* does not show distinct features in the visible region. The reduced *minus* oxidised spectra of the haem components of the purified oxidase complex from *E. coli* have been resolved from a set of spectra recorded at different redox potentials [91,100]. Cytochrome *b*-558 gives rise to an α-band at 560–562 nm in the absolute reduced and the reduced *minus* oxidised difference spectra, with the β-band at 531–532 nm. A small peak at around 595 nm is due to the α-absorbance of reduced haem *b*-595. In the *E. coli* oxidase, the resolved spectrum of haem *b*-595 also appears to exhibit a broad β-band in the 560 nm region [91,100]. The assignment of the 560nm-absorbance is important, e.g., for kinetic studies of electron transport (see [86]). Although the contribution of the haem *b*-595 β-band to the reduced *minus* oxidised difference spectrum at 560 nm may be as high as 50% [91,100], it should be born in mind that the band is broad and any changes of the sharp 560 nm peak can be assigned to haem *b*-558 only. The Soret features of the three haem components have not been resolved. However, there is some evidence from kinetic experiments that in the ferrous form haem *b*-558 absorbs near 429–430 nm and haem *b*-595 at 437–438 nm [101]. Ferrous cytochrome *d* is thought to exhibit only weak features in the Soret region [102,103]. This idea is supported by the small amplitude of the haem *d* CO binding spectrum [104,105]. Selected peak positions and extinction coefficients are summarised in Table 1.

Table 1
Extinction coefficients for cytochrome *bd* from *E. coli* and *A. vinelandii*

Spectrum	Organism	Haem	Wavelength pair (nm)	ϵ ($mM^{-1} \cdot cm^{-1}$)	Source
Reduced *minus* 'as prepared'	*E. coli*	*d*	628–649	18.8	[12]
			628–651	27.9 (pH 7.5)	[110]
	A. vinelandii		629–650	27.0	[16]
	E. coli	*d*	628–607	8.5	[64]
			628–608	7.4–8.0	[91]
	A. vinelandii		629–608	12	[16]
	E. coli	*b*-558 [a]	561–580	21.0	[110]
			560–580	14.8	[12]
	E. coli	*b*-595	595–606.5	1.9	[110]
CO/reduced *minus* reduced	*E. coli*	*d*	622–642	12.6	[12]
	A. vinelandii		622–642	18	[16]

The data are for pH 7.0 unless otherwise indicated

[a] The intensity of the 560-nm band is variable, depending on the purity of the preparation [16]. It may include spectral overlay by the β-band of haem *b*-595 (see Section 5.1).

Whereas cytochrome *bd* from *K. pneumoniae* and *Ph. phosphoreum* and the *appBC*-encoded *E. coli* enzyme have spectral features similar to those described above [13,14,24], the peak positions of reduced and ferrous CO-ligated cytochrome *d* are shifted up to 10 nm to the blue in purified [8,9] and membrane-bound [4–6] oxidases from *Bacillus* species.

5.2. EPR

In the anaerobically oxidised cytochrome *d* complex from *E. coli* two major high-spin species give rise to EPR signals [93,94]. One component of the signal has rhombic features with *g*-values near 6.3 (g_x) and 5.5 (g_y), the second, axial component appears at $g_{x,y} = 6.0$ and $g_z = 2.0$. On the basis of the correlation between the physicochemical properties of the EPR-detectable species and the optically defined cytochromes, the axial signal was attributed to cytochrome *d*, as were the weak rhombic signals in the high-field region at $g = 1.81$, 1.85, 2.3, 2.48 and 2.61. These represent low-spin sub-populations of haem *d* (together 15% of total haem *d*). The rhombic signal has been assigned to haem *b*-595. In air-oxidised ('as prepared') samples, a similar complex high-spin signal composed of rhombic and axial components is observed. However, under these conditions, haem *d* is in a ferrous, oxygen-ligated form (see below) and does not contribute to the signal. Both rhombic and axial components in 'as prepared' samples have been assigned to haem *b*-595 on the basis of ligand binding studies [16]. The lineshape of the composite *b*-595 signal shows a subtle dependency on the ligation state of haem *d* [93,106,107]. The axial form can be reversibly converted to the rhombic form, without optical or EPR spectral effects on haem *d*, by rising the pH [16,93] or addition of organic solvents such as ethanol or acetone (Jünemann and Wrigglesworth, unpublished results).

The EPR signal of the low-spin haem *b*-558 is difficult to observe. Only a weak, ramp-shaped g_z-feature at $g = 3.3$–3.4, part of a so-called HALS (highly anisotropic low spin) signal (see [108]), has been assigned to this redox centre [93,94,108]. The lability of the *b*-558 methionine axial ligand (see below) may cause a fraction of this haem being penta-coordinate, high-spin [108] which may be the rationale for the occasionally found assignment of the axial $g = 6$ signal to haem *b*-558 [109,110].

5.3. Haem midpoint potentials

The midpoint potentials of the redox centres in cytochrome *bd* from *E. coli* have been measured both optically and by EPR. Values reported for cytochromes *b*-595 and *d* are in good agreement, but more variation occurs in the published E_m of haem *b*-558 (Table 2). The latter is particularly sensitive to detergents [64]. Notably, the decrease in E_m of haem *b*-558 induced by cholate or octylglucoside is accompanied by some loss in quinol oxidase activity [111]. The midpoint potential of all haems in the complex is pH dependent between pH 6 and 10 with a $\Delta E_m/\mathrm{pH}$ of -60 mV for haem *d* and -30 to -40 mV for haems *b*-558 and *b*-595, indicating protonation events during reduction [64,94].

Isolated cytochrome *b*-558 [97] has very similar properties when compared to the *b*-558 compound in

Table 2
Midpoint potentials at pH 7 of the cytochromes in the *bd* complex from *E. coli*

Sample	E_m (mV)			Source
	b-558	*b*-595	*d*	
Membranes	165	147	260	[151]
Membranes	226	154	261	[93]
Purified *bd*, reconsituted in membranes	155	146	255	[64]
Membranes	195	136	260	[94]
Purified *bd* + 50 mM octylglucoside	61	113	232	[70]
Purified subunit I — in liposomes	160			[97]
Purified subunit I — 25 mM cholate	90			[97]

Table 3
Rate constants and dissociation constants of the reaction of cytochrome *bd* with oxygen and carbon monoxide at room temperature

	Oxygen			CO		
	k_{on} ($M^{-1} \cdot s^{-1}$)	k_{off} (s^{-1})	K_d (μM)	k_{on} ($M^{-1} \cdot s^{-1}$)	k_{off} (s^{-1})	K_d (μM)
E. coli	$2 \cdot 10^9$ [d]	50 [d]	0.025 [d]	$8 \cdot 10^7$ [d] 10^7 [a]	1.6 [d]	0.02 [d]
A. vinelandii	$2.8 \cdot 10^8$ [c]			10^8 [b]		

Source: [a] [104]; [b] [67]; [c] [105]; [d] [85].

the intact *bd* complex, in particular, as seen from Table 3, an unchanged value of E_m.

6. Haem ligation and active site structure

Hydropathy plots of the deduced protein sequence of the *E. coli* and *A. vinelandii* oxidases suggest several transmembrane helices in each subunit, seven in subunit I and eight in subunit II [20,22], a topology consistent with results from β-galactosidase and alkaline phosphatase gene fusion experiments [112,113], limited proteolysis [111,114] and protein crosslinking studies [115].

Subunit I has been implicated in the oxidation of ubiquinol. This was initially suggested from the inhibition of quinol oxidation by monoclonal antibodies raised against subunit I [3]. The treatment did not affect oxidation of TMPD. On the other hand, polyclonal antibodies against subunit II inhibited TMPD-oxidation but had no influence on ubiquinol oxidase activity. Hence, ubiquinol and TMPD bind at different sites, possibly located on different subunits. The epitopes of the monoclonal antibodies inhibiting quinol oxidation were mapped to a hydrophilic loop of 11 amino acids on the periplasmic side of the bilayer [116]. This region was therefore proposed to be part of the quinol binding domain. Limited proteolysis with trypsin or chymotrypsin results in selective cleaveage of subunit I at a single site close to the proposed quinol binding domain leading to a loss in ubiquinol oxidase activity without affecting TMPD oxidation [111,114]. The location of a Q-binding site on subunit I has been confirmed by stoichiometric binding of the radio labeled ubiquinone-analogue azido-Q (3-[^{3}H]azido-2-methyl-5-methoxy-6-geranyl-1,4-benzoquinone) to subunit I with subsequent loss of ubiquinol oxidase activity [117]. A second molecule of azido-Q, bound to subunit II, was ascribed to non-specific binding. A semiquinone from bound substrate has been described [118].

Apart from haem *b*-558, the axial ligands of the haems have not been identified at present. Only two of the ten histidine residues, His^{19} and His^{186} on subunit I, are essential for activity and haem ligation [119]. Whilst His^{186} has been determined as an axial ligand of haem *b*-558, mutations of His^{19} result in loss of both haems *b*-595 and *d*. Interestingly, this result suggests that both subunits are involved in binding of haems *b*-595 and *d*, since *cydB* mutants (no subunit II) also lack both cytochromes *b*-595 and *d* [19,97].

The axial ligands of haems *b*-595 and *d* are not known with certainty, and conflicting results have been reported. In an ENDOR study by Jiang et al. [120] no lines in addition to those due to pyrrole nitrogens were observed for an axial high-spin haem species ($g = 5.94$), taken to represent haem *d* the authors, hence indicating the absence of a strong nitrogenous ligand for this haem. However, the oxidation state of the oxidase is not clearly defined. Incubation of 'as prepared' enzyme with ferricyanide was used to generate the fully ferric enzyme. This method is now known to be not normally sufficient, since interaction of ferricyanide in absence of hydrophobic mediators appears to be kinetically impaired [121]. Hence, it is likely that haem *b*-595 in one of its alternative states (axial or rhombic) was observed. On the other hand, indication of a nitrogen axial ligand to haem *b*-595 comes from resonance Raman spectra on the basis of which His^{19} has been proposed as the ligand of haem *b*-595 [122]. The coordination of haem *d* is even less clear. A nitrogen axial ligand has also been proposed for haem *d* on the basis of hyperfine splitting of the EPR signal of ferrous, NO-ligated haem *d* [123]. If His^{19} is indeed

the ligand to haem *b*-595, then no histidines are left for haem *d* since the others appear to be non-essential by mutagenesis. Recent resonance Raman spectra of the haem *d*-cyanide adduct and the oxygenated and oxo-ferryl forms of haem *d* in 'as prepared' oxidase have been interpreted as showing the absence of any strongly coordinated amino-acid axial ligand for haem *d* [122,124]. The authors suggest that haem *d* has either no endogenous protein ligand or a weakly coordinated one that is easily displaced by exogenous ligands.

The finding that exogenous ligands such as CO can migrate between haems *b*-595 and *d* implies that these prostethic groups share a common binding pocket [83,125]. The narrow $\Delta\nu_{1/2}$ of the IR absorbance bands of the haem-CO adducts indicates a hydrophobic environment in the haem binding pocket [125].

Mutagenesis of the methionine residues in cytochrome *bd* has identified Met^{393} as the second axial ligand of haem *b*-558 [126]. This would imply close proximity of the transmembrane helices IV (His^{186}) and VI (Met^{393}). His-Met ligation of this haem has also been suggested from recent MCD and EPR studies [108].

Ligation of *b*-558 by His^{186} places this haem close to the putative quinol binding domain. Haem *b*-558 has previously been implicated in quinol oxidation. In mutant strains containing only subunit I, but lacking subunit II, reduction of haem *b*-558 by ubiquinol was observed although the protein did not show oxidase activity [97]. Interestingly, the His^{186} mutant does not oxidise TMPD [119]. This indicates that even though TMPD binds at a different site from ubiquinol (probably on subunit II or the subunit interface), electron transfer from this substrate still involves haem *b*-558.

EPR studies on oriented multilayer preparations of cytoplasmic membrane fragments of *E. coli* have revealed that haems *b*-558 and *d* are perpendicular to the membrane plane while haem *b*-595 is oriented at an angle of approximately 55° to the membrane plane [127]. From these data it can be proposed that haems *b*-558 and *d*, but not *b*-595, are bound between two transmembrane helices. Haem *b*-595, on the other hand, may be located outside the bilayer.

A model of the complex based on the above data is shown in Fig. 2. This model places the haems in two distinct active sites on opposite sides of the bilayer,

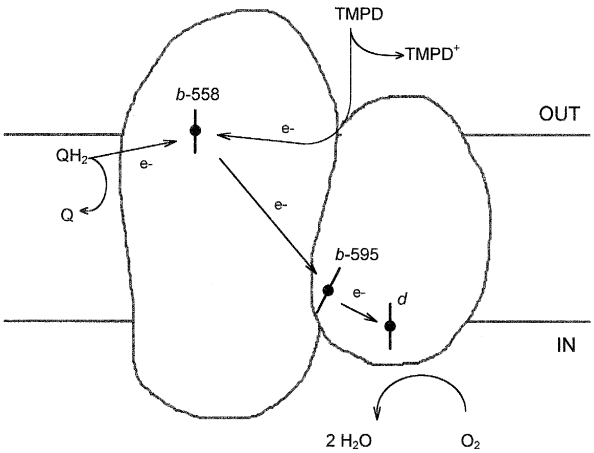

Fig. 2. Functional model of cytochrome *bd*. Based on [111]. The orientation of haems within the bilayer is drawn according to [127].

b-558 facing out towards the periplasm, the other haems binding near the cytoplasmic side of the bilayer. The topology of the oxidase as well as the proposed Q-site and haem ligands are also indicated in the sequence alignment in Fig. 1.

7. Reaction with ligands (other than oxygen)

Both pentacoordinate haems, *d* and to some extent *b*-595, bind small exogenous ligands, such as CO, cyanide or various nitrogen oxo-anions. Fully reduced oxidase has been used for most ligand binding studies, with only limited data on interaction of ligands with ferric haem *d*, largely because oxidised haem *d* does not show distinct features in the visible region of the spectrum. Mixed-valence compounds have been generated from 'as prepared' (= air-oxidised) enzyme. The interpretation of these latter data may be problematic due to the heterogeneity of the starting material with regard to redox/oxygenation state (see below).

7.1. Cyanide

Incubation of 'as isolated' cytochrome *bd* with cyanide results in decay of the oxygenated form. Since the cyanide-induced disappearance of the 650 nm peak is faster in the presence of respiratory substrate, it has been concluded that cyanide interacts

with a spectrally invisible ferric form of haem *d* [78,128]. The cyanide adduct itself has no prominent spectral features, but it does give rise to a low-spin EPR-signal at $g = 2.96–2.99$ [16,93,106]. Cyanide-induced changes to the EPR-spectrum of *A. vinelandii* oxidase also include some loss in intensity of the high-spin signal and, after prolonged incubation, a second weak low-spin signal at $g = 3.23$ [16,106] which have been taken to indicate some interaction of cyanide with haem *b*-595.

Krasnoselskaya et al. [129] note that the cyanide-induced decomposition of the cytochrome *d* oxy-compound, as monitored at 650 nm, can be kinetically correlated to a redshift in the Soret region. The authors interpret the redshift as a high-spin → low-spin transition of haem *b*-595 on cyanide binding and propose concerted binding of cyanide to ferric haems *b*-595 and *d*, possibly as a bridging ligand. This would conflict with the FTIR-evidence which points to a distance between a haem-bound CO molecule and the adjacent second haem iron greater than the Van der Waals contact distance [125]. The spectral changes of haem *b*-595 on cyanide binding to 'as prepared' cytochrome *bd* can also be interpreted as reduction of this haem during the transition of (b-595^{3+} d^{2+}-O_2) to (b-595^{2+} d^{3+}-CN^-), consistent with resonance Raman spectra of the cyanide adduct [124] and the loss in EPR intensity around $g = 6$ (see above).

Tsubaki et al. [110] also propose a cyanide bridge based on the position of the infrared band of the cyanide adduct and the complex behaviour of the EPR spectrum, including a novel $g = 3.15$ signal. However, the unusual IR frequency may be due to the chlorin structure of haem *d* or to its proposed pentacoordinate state in presence of an exogenous ligand [124]. Tsubaki et al. [110] do not account for the electron released from haem *d* upon cyanide binding to 'as prepared' enzyme.

Cyanide inhibits reduction of haem *d*, but not of the *b*-type haems, by quinol or dithionite [16,130]. This is consistent with the finding that, in purified *A. vinelandii* oxidase, cyanide (10 mM) lowers the midpoint potential of haem *d* from around 240 mV to less than −150 mV but does not affect the E_m value of haem *b*-595 (Jünemann and Rich, unpublished data). The latter observation also indicates that cyanide can not be a strong ligand of haem *b*-595. The conclusion that cyanide does not bind to haem *b*-595 has also been reached from resonance Raman spectroscopy [124].

Cyanide binding to ferrous haem *d* has been found to be negligible [130].

7.2. Carbon monoxide

CO binding to fully reduced cytochrome *bd* induces a shift of the haem *d* α-peak from 630 nm to 636 nm. This is reflected, in the CO difference spectrum, by a trough near 623 nm and a peak near 644 nm. In the Soret region, CO binding to haem *d* gives rise to troughs at 443 nm and 415–426 nm, with similar amplitude as the α-band signal [16,104,105]. The feature at 443 nm has previously been tentatively assigned to the CO compound of haem *b*-595, primarily on the grounds that haem *d* is thought to contribute little to the Soret absorbance [102,103], but is now, on the basis of CO recombination studies, recognised to be due to the CO adduct of haem *d* [104,105]. CO binding to haem *b*-595 however has been clearly demonstrated by FTIR at low temperatures [125] and by CO recombination after flash photolysis at cryogenic [131] and room temperature [104,105], although the interaction occurs with low affinity [16] and is not strong enough to increase the midpoint potential of haem *b*-595 [93].

Some purified preparations of cytochrome *bd* show a trough at 560 nm in the CO difference spectrum [91,104], suggesting that some CO binding to haem *b*-558 may also take place, possibly due to denaturation/lability of the methionine axial ligand during the isolation procedure.

At least three (in *E. coli* oxidase, [104]) or four (in *A. vinelandii* enzyme, [105]) phases of CO recombination to dithionite-reduced cytochrome *bd* have been resolved at room temperature. Only one of these is due to the haem *d*-CO compound and the remaining, slower, phases have been ascribed to reaction of CO with the *b*-type haems. The rate constant of CO binding to haem *d* is in the region of 10^7 $M^{-1} \cdot s^{-1}$ to 10^8 $M^{-1} \cdot s^{-1}$ (see Table 3), thus approaching the limit for a diffusion controlled reaction. Whilst CO-trapping by haem *b*-595 has been demonstrated at 4K [125], no indication of intermediate CO binding to this haem following photolysis from haem *d* has been observed at room temperature.

In *A. vinelandii*, CO has been shown to displace oxygen from haem *d* in 'as prepared' oxidase, resulting in a mixed-valence CO compound where both *b*-type haems remain oxidised (b-558^{3+} b-595^{3+} d^{2+}-CO) [82,88]. The haem *d* α-band is also positioned at 636 nm. The broader bandwidth compared to the fully ferrous CO compound and the partial shift of the 650 nm peak of oxygenated haem *d* to 639 nm observed by Kauffman et al. [82] can be attributed to an incomplete conversion of the oxy- to the CO species. The photolysis spectrum of the haem *d* CO compound in mixed-valence oxidase is identical to that in the fully reduced enzyme [88], although the recombination rate constant is 40% lower than in fully ferrous enzyme [88], possibly due to some electron backflow following photolysis.

Under turnover conditions, haem *d* does also form a CO adduct with an α-band at 639 nm [82]. Since the oxo-ferryl compound absorbing at 680 nm is still present, the spectrum is likely to represent a steady-state mixture of the CO compound and oxygen intermediates, suggesting that a high rate of CO dissociation (two orders of magnitude higher than in bovine heart cytochrome *c* oxidase, see [85] and references therein) allows turnover to proceed in a small proportion of unligated enzyme rather than via haem *b*-595 as an alternative oxygen binding site.

7.3. Nitrogen-containing ligands

Interaction of the ferrous enzyme with nitrogen containing ligands has been investigated in membrane-bound cytochrome *bd* from *A. vinelandii* [82] and *E. coli* [81,92] and, more recently, in purified oxidase from both organisms [16,123,132]. NO, NO_2^-, $N_2O_3^{2-}$ and NH_2OH induce a shift of the α-peak of reduced haem *d* in membrane preparations to 642 nm. The common peak position has been interpreted as all ligands giving the same or a very similar haem-nitrosyl compound [82]. Similarly, identical EPR spectra of a haem *d*–nitrosyl compound have been obtained when dithionite-reduced oxidase was incubated with NO, nitrite [123], nitroxyl or trioxodinitrate (Jünemann, Khan and Hughes, unpublished data). In the case of trioxodinitrate the reacting species is thought to be the nitroxyl ion which is produced during self-decomposition of trioxodinitrate ($N_2O_3^{2-} \rightarrow NO^- + NO_2^-$). Rothery et al. [81] kinetically resolved two nitrite-binding haems within the oxidase complex, namely haem *d* (identified from its α-peak) and a slower species giving rise to a trough at 438 nm in the nitrite difference spectrum. The latter was attributed to haem *b*-595, on the grounds that parallel but very weak spectral changes were observed around 600 nm. This supports the previous assignment of the shoulder on the red side of the Soret peak in the reduced *minus* air-oxidised spectrum to haem *b*-595 (see [133]). Although an earlier report has described biphasic kinetics of NO_2^- or NO^- binding to haem *d* [92], quantitative EPR as well as kinetic experiments show that a single haem *d* is involved in binding of nitrosyl ligands ([16,81], Jünemann, Khan and Hughes, unpublished data), consistent with the haem stoichiometry.

The nitrosyl-haem EPR signal around $g = 2$ is composed of two distinct species, indicating two different NO binding sites, haem *d* and haem *b*-595 [16,123]. Both signals, that of haem *d*-NO at stoichiometric amounts of nitric oxide and that of nitrosyl–haem *b*-595 at excess NO, quantitate to one spin/site each per complex, indicating the absence of spin coupling. The signal of the haem *d*-NO compound has been shown to have a hyperfine structure indicative of a nitrogen axial haem ligand [123].

As judged from a shift of the haem *d* α-peak to 642–644 nm, NH_2OH, NO and NO_2^- replace O_2 in 'as prepared' enzyme, leading to mixed-valence nitrosyl compounds [82]. In this case, the NO-adduct, but not the nitrite compound, shows a $g = 2$ EPR signal [132]. The lineshape of this signal is more axial than in fully reduced oxidase [123,132]. NO-binding to haem *d* in the mixed-valence enzyme also involves subtle lineshape changes of the haem *b*-595 high-spin EPR signal.

Under turnover conditions, hydroxylamine induces a partial blueshift of the haem *d* α-peak, with an increase in bandwidth [82] which can be interpreted as showing a mixture of nitrosyl and oxygenated (turning over) haem *d*, similar to the findings using CO.

Azide shows no effect on the air-oxidised enzyme unless respiratory substrate is present [82]. Then, the haem *d* α-band is shifted to 655 nm, accompanied by partial inhibition of turnover. On anaerobiosis, the α-peak of reduced cytochrome *d* appears at its normal position at 630 nm, suggesting that azide reacts

with ferric but not ferrous cytochrome *d* and that azide binding to haem *d* is not strong enough to prevent its reduction. Azide binding to ferric haem *d* has also been implied by the loss of the low-spin EPR signals in the $g = 2.3–2.6$ region [134].

7.4. Hydroxide

Exposure of cytochrome *bd* to hydroxide (pH 9) results in a reversible decay of the oxygenated form of haem *d*, as judged from a loss of the α-peak near 650 nm [16]. A redshift in the Soret region (trough at 416 nm and peak at 432 nm in the pH9 *minus* pH7 difference spectrum) and signals at 560 nm and around 590–600 nm are also observed (Jünemann and Wrigglesworth, unpublished results). These findings can be interpreted as hydroxide-induced generation of ferric haem *d*, accompanied by internal electron redistribution leading to partial reduction of both *b*-type haems. There is no EPR or optical evidence, though, for the formation of a haem *d*-hydroxide adduct. However, exposure to alkaline pH does induce a major effect on the *b*-595 high-spin EPR signal around $g = 6$, namely a reversible lineshape change from predominantly axial to predominantly rhombic which is not kinetically correlated to the decay of the haem *d* oxy-form, and some loss in signal intensity [16]. The latter is compatible with partial reduction of haem *b*-595. The decay of the oxygenated haem *d* compound under alkaline conditions may be due to disruption of a hydrogen bond to a distal amino acid residue stabilising the haem-oxygen adduct. Exposure to OH^- would leave the complex essentially in the unligated mixed-valence form where electron redistribution occurs due to pH-induced shifts of the haem redox potentials. However, OH^- does not appear to act as haem ligand, and the interpretation of the observed pH-effects is still an area of uncertainty.

7.5. Overview

Overall, cytochrome *bd* contains one high-affinity site for exogenous ligands, i.e., haem *d*, with haem *b*-595 providing a second, low-affinity and/or kinetically slower site. Under non-turnover conditions, the contribution of haem *b*-595 to ligand binding appears to be minor as judged from, e.g., the lack of an E_m effect with CO or CN^-, or the non-saturating CO binding at ≤ 1mM CO. Stoichiometric binding is only observed with excess levels of a highly reactive ligand, NO. It is not known whether transient ligand binding is an obligatory step during catalysis (see below). Although haem *b*-558 is hexacoordinate and would not normally have a ligand binding role, some effects have been ascribed to this haem by Borisov [135] on the basis of MCD. However, the spectral features of the low-affinity/slow ligand binding site, in particular for CO or NO, clearly point to haem *b*-595 as providing this site. Ligand binding by haem *b*-558 (e.g., as observed in [91]) appears to be solely due to a partially denatured oxidase preparation.

8. Reaction with oxygen and electron transfer pathway

8.1. Oxygen-intermediates

Two stable oxy-forms of cytochrome *d* have been identified, an oxygenated (Fe^{II}-O_2, absorbing near 650 nm) and an oxoferryl ($Fe^{IV} = O$, absorbing near 680 nm) species [95,98,99]. Membrane and purified preparations can be seen to be a mixture of these two forms, with at least 70% oxygenated enzyme [16,129,136], plus some fully ferric enzyme, which may appear only as a freezing or purification artefact [94].

The d^{2+}-O_2 species is similar to oxymyoglobin, as demonstrated by resonance Raman spectroscopy [99]. The oxygenated compound is formed by reversible binding of oxygen to the one-electron reduced enzyme [136]. It has been proposed that the oxygenated species (b-558^{3+} b-595^{3+} d^{2+}-O_2) is not an intermediate in the main catalytic cycle (see below).

The intermediate absorbing at around 680 nm has previously been interpreted as a peroxy-compound of the type Fe^{II}-O_2^{2-} [136,137], since it can be obtained by reaction of excess H_2O_2 with haem *d* in 'as prepared' enzyme ($b^{3+}b^{3+}$ d^{2+}-O_2). However, resonance Raman spectroscopy indicates that scission of the oxygen–oxygen bond has already taken place, and the species has been identified as an oxo-ferryl form [95]. A spectrally identical compound is formed on reaction of ferric enzyme with H_2O_2, invoking a one-electron reduction by either H_2O_2 or an endoge-

nous donor [138,139]. This reaction is reversible by addition of catalase, however at a rate by an order of magnitude slower compared to the expected value k_{off} for peroxide calculated from the K_d (30 μM) and the k_{on} (600 $M^{-1} \cdot s^{-1}$) and assuming a peroxy, not an oxo-ferryl species [138,139]. This release of the oxygen molecule from an oxo-ferryl compound may be analogous to the oxygen exchange with bulk water observed in compound II of various peroxidases [140–142].

H_2O_2-binding experiments with 'as prepared' oxidase were interpreted as showing the formation of two distinct species, named the 'peroxy-intermediate' at substoichiometric amounts of peroxide and the 'peroxide-state' at excess H_2O_2 [136]. However, interaction of peroxide with fully ferric cytochrome *bd* appears to generate only one species, with the spectral signature of the so-called 'peroxy-intermediate' [138,139]. Only the formation of the 'peroxide state' from 'as prepared' enzyme is accompanied by decay of the 650 nm-band of oxygenated haem *d*. Hence, the biphasic reaction [136] is likely to represent reaction of H_2O_2 with ferric and oxygenated enzyme in a preparation heterogeneous with respect to redox state and oxygen ligation of haem *d* (see above).

8.2. Electron transfer

Direct measurements of internal electron transfer have been carried out using the flow/flash technique [143] and its low-temperture equivalent, followed optically and by EPR. There are generally no quantitative data from the low-temperature experiments, but qualitatively similar results are obtained as at room temperature. The kinetics of the two distinct *b*-type haems have not been resolved.

Reaction of fully ferrous cytochrome *bd* with oxygen results in an oxy-adduct (b-558^{2+} b-595^{2+} d^{2+}-O_2, absorbing at 650 nm) as a first product which decays to give rise to the 680 nm compound [85,98]. The transition from the oxygen-bound to the oxo-ferryl form occurs at 10^4 s^{-1} at room temperature in the *E. coli* enzyme [85]. This is kinetically correlated to *b*-haem reduction, monitored at 560 nm, a spectral region dominated by haem *b*-558.

Low-temperature studies of aerobic oxidation of cytochrome *bd* suggest that haem *b*-595 may be oxidised before haem *b*-558 since a Soret absorbance at 437 nm and a feature near 590 nm are fully developed before their counterparts at 430 nm and 562 nm [98]. EPR studies [109,144] concentrate on the low-field region of the spectrum, i.e., showing only the high-spin haem signals due to *b*-595/*d*, but not the $g = 3.3–3.4$ feature of haem *b*-558. However, an interesting result of these experiments is that development of the axial and rhombic components of the high-spin haem signal does not occur simultaneously, but that the rhombic species precedes the axial one(s). Bearing in mind that the reaction proceeds *via* an EPR-silent haem *d*-oxy adduct to end at an equally EPR-silent oxo-ferryl form, but not ferric *d*, it can be assumed that the $g = 6$ region is dominated by haem *b*-595. Hence, the axial and rhombic signals of haem *b*-595 may be associated with alternative configurations of the haem pocket in different catalytic intermediates.

Although electron transfer rates to/from haem *b*-595 have not been determined, a simple linear electron transfer scheme of the order b-558 → b-595 → d → O_2 has been proposed based on low-temperature work [109].

Reversed electron transfer has been measured in decyl-aurachin D-treated, mixed-valence CO-ligated oxidase from *A. vinelandii* [88]. The presence of decyl-aurachin D leads to a shift of the *b*-haem midpoint potentials resulting in an increased amplitude of electron backflow following photolysis of the CO compound. The inhibitor does not appear to affect the rate of reversed electron transfer. Oxidation of haem *d* and concomitant reduction of haem *b*-558 occur at a rate constant of about 3000 s^{-1}.

8.3. Catalytic cycle

Based on the identification of some oxygen intermediates [95,99] and the enzyme kinetics during turnover with ubiquinol-1 (plus DTT) a catalytic cycle, shown in Fig. 3, has been proposed for cytochrome *bd* [67]. The enzyme turns over via the one- and three- electron loaded forms, without invoking a fully ferric species. Since the flow-flash experiment ends at the oxo-ferryl enzyme (8 to 3 in Fig. 3) there is no indication of more than three reducing equivalents in the fully ferrous enzyme [85]. Under normal turnover conditions, ubiquinol binds to the one-electron reduced enzyme (5), followed by oxy-

gen binding to ferrous haem *d* (6). Scission of the oxygen–oxygen bond and release of one molecule of water lead to the formation of the oxo-ferryl form of the enzyme (3). The presence of three reducing equivalents in species (1) facilitates this reaction, leaving the enzyme in a 'Compound II' form as found in the catalytic mechanism of peroxidases. The enzyme then accepts two further electrons from ubiquinol and, following the release of a second molecule of water, returns to the one-electron reduced form. Although it can be inferred from the relative midpoint potentials of the three haems (Table

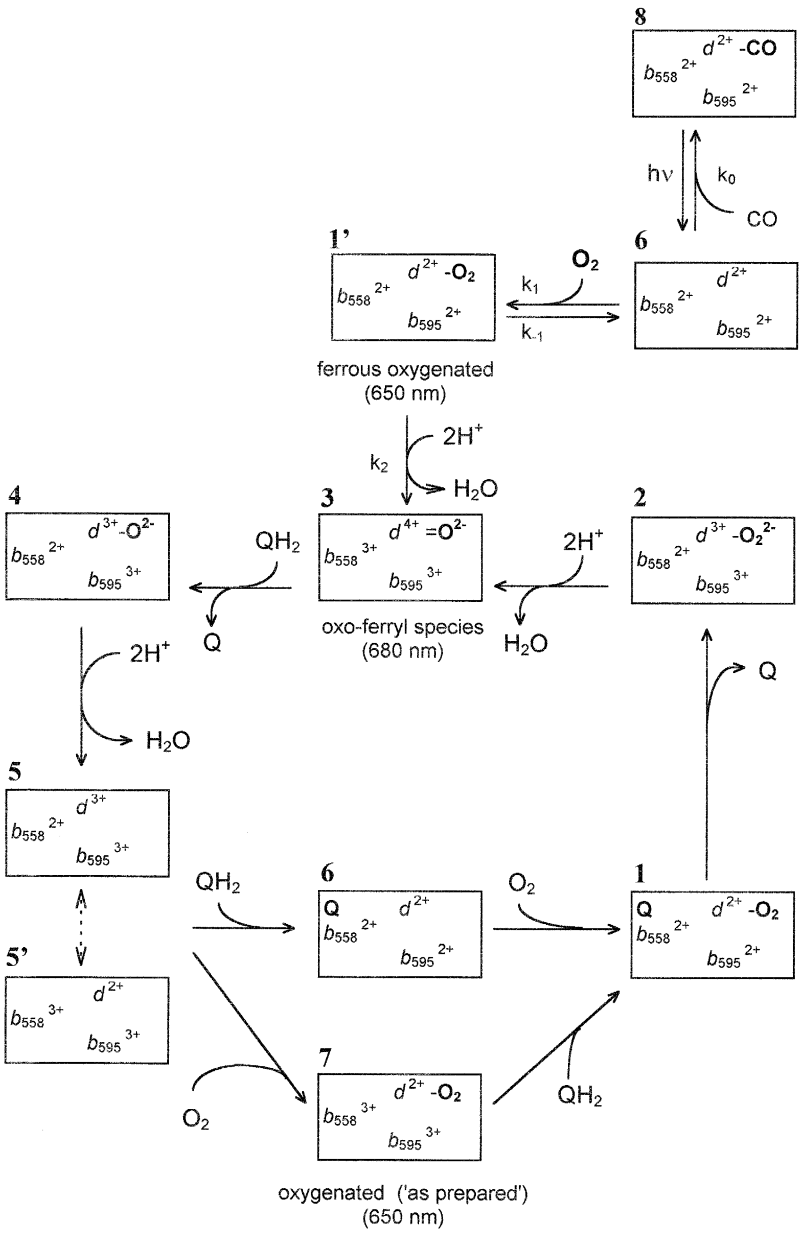

Fig. 3. Proposed catalytic cycle and reactions of ferric cytochrome *bd* with O_2 and CO. Intermediates 1–7 represent the catalytic cycle proposed in [67]. The oxo-ferryl (3) and oxygenated (7) species are those found in membrane-bound and purified preparations of the oxidase. The cycle also includes hypothetical peroxy and hydroxy intermediates. Intermediates $8 \rightarrow 6' \rightarrow 1' \rightarrow 3$ represent events during a flow-flash experiment [85]. The rate constants for reactions $8 \rightarrow 6'$ and $6' \rightarrow 1$ are found in Table 3. The rate constant for the formation of the oxo-ferryl intermediate ($1' \rightarrow 3$) is $k_2 = 10^4\ s^{-1}$ [85].

2) that in the one-electron reduced form (5/5′) the electron resides mostly on haem *d*, an intermediate such as 5 with ferric haem *d* must be populated during the steady-state since the rate of cyanide binding is enhanced during turnover (see above). The scheme shows the fully ferrous enzyme (6) as the major oxygen-binding species, and the mixed-valence O_2-compound (7) is not included in the main cycle but is formed at low levels or in the absence of ubiquinol. Support for this model comes from the observation that in oxygen-electrode assays full oxidase activity is developed only after a lag phase when the reaction is started with oxygenated enzyme whereas no lag phase is observed with oxidase in the ferrous or oxo-ferryl states [145]. As yet it is not known why the substrate binding kinetics should be so different in the two branches as to favour step $5/5' \rightarrow 6$ over $5/5' \rightarrow 7$. However, binding of CO to haem *d* is 40% slower in the mixed-valence form [88], and it could be surmised that oxygen-binding may follow a similar pattern. Clearly, further work is needed to fully resolve the kinetics of the catalytic cycle.

In *E. coli* the fully reduced enzyme, postulated to be the major oxygen binding form during turnove, has a similar K_D of about 20 nM for oxygen and CO (Table 3). This is much lower than the operational oxygen affinity (K_m) which is in the μM region. Hill et al. [85] have pointed out that this is a factor distinguishing the *bd*-type oxidases from the haem-copper oxidases. In the latter, rapid electron transfer following oxygen binding lowers the K_m (~ 1 μM) relative to the K_D (~ 40 μM) and leads to kinetic trapping of oxygen in an oxo-ferryl intermediate. In contrast, cytochrome *bd* which shows rapid and high-affinity O_2 binding can be described essentially as a thermodynamic trap for oxygen.

9. Role of haem *b*-595 — a binuclear centre?

Based on the observation of CO trapping on haem *b*-595 following photolysis of the haem *d*-CO adduct at 4K it has been suggested that these two haems form a binuclear centre analogous in function to the haem-copper binuclear centre of the proton-pumping oxidases [125]. However, it has since emerged that there can not be an extensive analogy to the haem-copper binuclear centre, and it is questionable whether the term ‘binuclear centre’ can be applied to cytochrome *bd*. Most importantly, no spin coupling between haems *b*-595 and *d* has been oberved [16]. Cyanide binding in the presence of substrate is only three [16] to 50-fold [78] faster than under non-turnover conditions. This increase has been ascribed to generation of some ferric haem *d* during turnover, in contrast to mitochondrial cytochrome *c* oxidase where the rates of cyanide binding to the oxidised and the partially reduced enzyme differ by a factor of 10^6 due to a strong redox interaction between haem a_3 and Cu_B [80]. Although some redox interaction between haems *b*-595 and *d* has been noted by Meinhardt et al. [94], ligation of haem *d* to CO [93] or cyanide (Jünemann and Rich, unpublished data) does not alter the redox potential of haem *b*-595.

On the other hand, the observation of ligand migration [83,125] suggests that haems *b*-595 and *d* do share a binding pocket, a fact supported by the finding that all *b*-595 deficient mutants to date also lack haem *d* [119,146]. This would be consistent with some conformational interaction as manifested by a subtle dependency both of the lineshape of the *b*-595 EPR signal around $g = 6$ on the ligation state of haem *d* [93,106] and of the lineshape of the d^{2+}-NO EPR signal around $g = 2$ on the redox state of haem *b*-595 [123,132].

At present, little is known about the function of haem *b*-595 in the oxidase mechanism. Although under non-turnover conditions ligand binding to haem *b*-595 is slow and with low affinity, several lines of evidence point to some ligand binding role of haem *b*-595 during turnover. Photochemical action spectra showing light relief of CO-inhibited samples have a clear peak around 590–600 nm [43,147]. A substantial residual activity is observed in presence of approximately 1 mM CO [43,82]. As discussed above, this may be due to the high dissociation rate constant of the haem *d*-CO adduct since at least one steady-state oxygen intermediate, the oxo-ferryl form,can still be clearly detected optically. However, it can also be speculated that in the presence of CO turnover might proceed via haem *b*-595 as an alternative oxygen binding site. Oxygen binding by haem *b*-595 with CO-ligated haem *d* has been suggested by Poole’s group [69,83]. Rothery et al. [81] have observed some cooperativity during nitrite-inhibited

oxygen consumption with an apprent number of oxygen-binding sites of $n > 1$. Although this finding may indeed reflect oxygen binding to both haem *d* and haem *b*-595 during turnover, it should be noted that apparent cooperative behaviour has also been reported for bovine heart cytochrome *c* oxidase where the effect can be ascribed to changes in the ratio of the rate constants of various electron transfer steps in a system which does not obey simple Michaelis–Menten kinetics [148,149].

From the pH-dependency of the reaction of nitrite with haem *b*-595 [81] and the sensitivity of its EPR signal to pH [16] it may be concluded that a protonation site is close to haem *b*-595 which may be involved in providing substrate protons.

If ligand binding to haem *b*-595 is indeed an obligate step during catalysis, the mechanism will still be very different from the processes at the haem-copper binuclear centre. In the haem-copper binuclear centre, the Cu_B acts as a gateway with oxygen binding to the copper preceding ligation to the haem, as is reflected, e.g., in saturation kinetics of ligand binding (see [150] for a review). This contrasts the situation in cytochrome *bd* where haem *b*-595 appears to have a less pronounced role [85] and may only act as a secondary oxygen-binding site.

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