Changes in the redox state and composition of the quinone pool of *Escherichia coli* during aerobic batch-culture growth

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Ubiquinones (UQs) and menaquinones (MKs) perform distinct functions in *Escherichia coli*. Whereas, in general, UQs are primarily involved in aerobic respiration, the MKs serve as electron carriers in anaerobic respiration. Both UQs and MKs can accept electrons from various dehydrogenases, and may donate electrons to different oxidases. Hence, they play a role in maintaining metabolic flexibility in *E. coli* whenever this organism has to adapt to conditions with changing redox characteristics, such as oxygen availability. Here, the authors report on the changes in both the size and the redox state of the quinone pool when the environment changes from being well aerated to one with low oxygen availability. It is shown that such transitions are accompanied by a rapid increase in the demethylmenaquinone pool, and a slow increase in the MK pool. Moreover, in exponentially growing cultures in a well-shaken Erlenmeyer flask, it is observed that the assumption of a pseudo-steady state does not hold with respect to the redox state of the quinone pool.

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INTRODUCTION

The quinones serve as membrane-associated electron carriers in the respiratory chain that carry out electron transfer between various electron donors and acceptors. Ubiquinone (UQ)-8 (coenzyme Q₈ or UQ₈) is the main quinone in *Escherichia coli* involved in aerobic respiration, and in electron transfer towards nitrate (Brondijk *et al.*, 2002; Soballe & Poole, 1999; Wallace & Young, 1977). Under anoxic conditions, but in the presence of other electron acceptors, either demethylmenaquinone (DMK)-8 (DMK₈) or menaquinone (MK)-8 (MK₈) serves as the main electron-transferring quinone. Whether the cell uses DMK₈ or MK₈ depends on the particular carbon source and electron acceptor present in the growth medium (Unden, 1988; Wissenbach *et al.*, 1992).

It is well-known that *E. coli* is endowed with a highly versatile respiratory chain that allows it to adapt rapidly to conditions that may vary to a great extent with respect to

their redox state (de Graef *et al.*, 1999; Poole & Cook, 2000; Smith & Neidhardt, 1983). In order to achieve this flexibility, synthesis and turnover of both UQs and MKs should be rapid upon changing conditions. It has been shown that control of the composition of the quinone pool does not take place at a transcriptional or translational level, but rather occurs at the post-translational level of the access enzymes in the MK₈ biosynthetic pathway (Shestopalov *et al.*, 1997). This form of regulation implies that UQ₈ and MK₈ production can be effected within minutes. Our previous work has elucidated some of the subtleties that underlie the control of electron flow through the alternative pathways of the respiratory chain (Alexeeva *et al.*, 2002; Calhoun *et al.*, 1993). Here, we present data that illustrate the sensitivity of the chain to external redox conditions by use of a single and rapid method that determines the content of the quinone pools and the redox state of the UQ pool. It is shown that the assumption of a pseudo-steady state during the exponential growth phase in a standard experimental set-up with a rotary shaker is not correct with respect to the composition and functioning of the respiratory chain. The synthesis of

Abbreviations: DMK, demethylmenaquinone; MK, menaquinone; UQ, ubiquinone.

DMK₈ during transitions from high to low oxygen tensions is discussed.

METHODS

Strains and growth conditions. *E. coli* strain MC4100 was used throughout this study. A $\Delta ubiE$ mutant derived from JC7623, and called JC7623 $\Delta 4$ -1, which was kindly provided by C. Clarke (Lee *et al.*, 1997), was also used. Cells in batch cultures were grown in Evans medium (Evans *et al.*, 1970) (pH 6.9 ± 0.1) at 37 °C. Sodium phosphate was used as a buffer at a concentration of 100 mM. Glucose, selenite and thiamine were added to final concentrations of 50 mM, 30 $\mu\text{g l}^{-1}$ and 15 mg l^{-1} , respectively. Aeration was accomplished by growing 30 ml culture volumes in 300 ml Erlenmeyer flasks, or 100 ml volumes in 1 l Erlenmeyer flasks, in a rotary shaker (Gallenkamp Orbital Incubator) set at 200 r.p.m. Cells were taken from cultures grown aerobically overnight, and inoculated at an OD₆₀₀ of 0.1.

Cells grown in chemostat conditions were grown under glucose-limited conditions (Bioflo III) at a constant dilution rate of $0.35 \pm 0.01 \text{ h}^{-1}$ in a vessel containing 1200 ml culture. Glucose (10 mM) was used as the single carbon and energy source. Selenite and thiamine were added to final concentrations of 30 and 15 mg l^{-1} , respectively. pH was maintained at 7.0 ± 0.1 by titrating with sterile 1 M NaOH, and temperature was set to 35 °C. Oxygen supply was regulated by a Brooks mass flow controller 5850 E series. The air supply was set at 1200 ml min⁻¹. Anaerobic shift was performed by changing the input gas from air to nitrogen. Residual dissolved oxygen was monitored by an Ingold polarographic oxygen sensor, with a detection limit of 100 nM.

Quinone extraction and analysis. Samples from chemostat cultures were taken by use of a sampling device (Lange *et al.*, 2001) to ensure reproducible and quantitative aliquots within 0.5 s. Samples from batch cultures were taken by pipetting within 2 s. Samples (2 ml) were quenched with 6 ml ice-cold 0.2 M HClO₄ in methanol. Petroleum ether (6 ml; 40–60 °C) was then added rapidly to the mixture, and vortexed for 1 min. After the mixture was centrifuged (900 g, 2 min), the upper petroleum ether phase was removed, transferred to a test tube, and evaporated to dryness under a flow of nitrogen. Another 6 ml petroleum ether was added to the lower phase, and the vortexing and centrifugation steps were repeated. The upper phases were combined. After evaporation to dryness, extracts could be stored for at least 7 days under nitrogen at -20 °C without any detectable auto-oxidation. Immediately before use, the extracted UQ/ubiquinol was resuspended with a glass rod in 80 μl ethanol and analysed in a HPLC system (Pharmacia LKB gradient pump 2249 system, with an LKB 2151 variable-wavelength monitor) containing a reverse-phase Lichrosorb (Chrompack) 10 RP 18 column (4.6 mm i.d., 250 mm length). The column was equilibrated with ethanol : methanol (1 : 1, v/v), and this mixture was used as the mobile phase. The flow rate was set at 1 ml min⁻¹. Detection of the quinones was performed at 290 nm for UQs, at 248 nm for MKs, and at 270 nm to record all quinones simultaneously. The amounts of all quinones were calculated from the peak areas using UQ₁₀ and MK₄ as standards, according to the method applied by Shestopalov *et al.* (1997). Methanol, ethanol and petroleum ether were of analytical grade.

Quinone identification. Peaks were identified by UV/Vis spectral analysis and mass spectral analysis. A UV/Vis spectrum of DMK₈ was kindly provided by Dr Bogachev (Moscow State University, Russia). For mass spectral analysis, fractions collected from the HPLC were evaporated under nitrogen, and redissolved into 89% acetonitrile, 10% water and 1% formic acid (v/v; LC-Grade; Merck). The fractions were analysed by off-line nano-electrospray MS using coated

PicoTips (Econo12; New Objective) on an electrospray ionization QTOF mass spectrometer (Micromass). Ions selected for MS/MS collided with argon in the hexapole collision cell. Data processing and evaluation were performed using Masslynx software (Micromass).

RESULTS

Method for determination of the reduction state and content of the quinone pools

A method that has been used to determine the reduction state of the ubiquinone pool in *Paracoccus denitrificans* (Otten, 2001; Van den Bergen *et al.*, 1994) was adapted for determining the reduction state of the quinone pool in *E. coli*. Quinones were isolated as described (see Methods), and separated by HPLC. The UV/Vis spectra of all peaks shown in the chromatogram (Fig. 1) were recorded (shown at 270 nm). The peaks at 8 and 12.5 min elution times showed spectra typical for UQ₈H₂ and UQ₈. Chromatograms of quinone isolations from the $\Delta ubiE$ mutant, which is unable to catalyse the final methylation step for formation of UQ (Lee *et al.*, 1997; Wissenbach *et al.*, 1992), did not show these two peaks (Fig. 1). Mass spectral analysis showed the presence of the correct masses for UQ₈H₂ and UQ₈ (729.5 and 727.5 m/z , respectively). In addition, MS/MS fragmentation of selected UQ peaks showed tropylium ions at 197.1 m/z . Formation of these tropylium ions is specific for fragmented UQs (Geyer *et al.*, 2004).

Fig. 1. HPLC analysis of the quinone pool of *E. coli*. Extracts from an *E. coli* MC4100 (wild-type) and JC7623 $\Delta 4$ -1 ($\Delta ubiE$) culture were fractionated by reverse-phase HPLC. The mixture of quinones was analysed by UV/Vis absorption spectroscopy at 270 nm (see Methods).

The peaks at 18 and at 21 min showed UV/Vis spectra typical for DMK₈ (reference spectrum kindly provided by Dr Bogachev) and MK₈. Mass spectral analysis showed the presence of the masses of DMK₈ and MK₈ (703.6 and 717.6 *m/z*, respectively). Confirmation by MS/MS (173.1 and 187.1 *m/z* for DMK₈ and MK₈, respectively; Geyer *et al.*, 2004) showed the expected fragmentation products. Quinone samples isolated from the $\Delta ubiE$ mutant (Lee *et al.*, 1997), which is also impaired in the methylation of DMK to MK, did show a peak at 18, but not at 21 min. From these data, it can be concluded that the peaks at 18 and 21 min are due to the presence of DMK₈ and MK₈, respectively, and that the peaks identified in Fig. 1 consist solely of the indicated quinones. Peaks for reduced DMK₈ and MK₈ were found to elute around 6 and 7 min, respectively (data not shown). These reduced forms, however, always showed nearly complete oxidation during quinone isolation, and are therefore not included in this report. Hence, it is assumed that the determined DMK₈, MK₈, represents the total of oxidized plus reduced DMK₈, i.e. the total of oxidized plus reduced MK₈.

Effect of the length of the sampling time: kinetics of DMK₈ production

When the size of the UQ pool is known (in moles per cell mass), together with the respiration rate (in moles of oxygen reduced per unit time per cell mass), the turnover of the UQ pool can be calculated. Thus, in rapidly aerobically growing *E. coli* cells (μ 0.7 h⁻¹) with a specific respiration rate of 10 mmol h⁻¹ g⁻¹ (Calhoun *et al.*, 1993), and a UQ content of 1 μ mol g⁻¹ (see Fig. 6), the turnover of the UQ₈ pool is approximately 180 ms. Hence, fast

Fig. 2. The effect of restricted oxygen availability on the reduction state of the UQ₈/UQ₈H₂ pool of *E. coli*. Samples were directly quenched in a methanol/HClO₄ solution to minimize alterations during the isolation procedure. The x-axis shows the time points of sampling after a shift to low aeration following aerobic growth to an OD₆₀₀ of 3.0. White bars, total UQ₈ + UQ₈H₂; grey bars, UQ₈H₂; black bars, UQ₈. Error bars indicate SD.

quenching of the sampled cells is essential to obtain representative data regarding the redox state of these pools. The redox state of the quinone pool was analysed in time, with a frequency of 5 s, when a mid-exponential culture with a biomass concentration of 1 g dry weight l⁻¹ was transferred from aerobic well-shaken conditions to conditions without shaking (i.e. the oxygen supply was limited by the rate of oxygen diffusion). The redox state of the UQ pool remained constant for at least 5 s, since no increase in amount of UQ₈H₂ was observed in the first 5 s. However, within 10 s, the UQ₈ pool showed a significant reduction that lasted until 40 s after limitation of the oxygen supply (Fig. 2). Care was taken to quench all further samples within 5 s.

Over the analysis period, the total content of UQ₈ did not change significantly upon restriction of the oxygen supply. In contrast to this, the DMK₈ content showed a large increase during the first 40 s (Fig. 3). No significant increase in MK₈ content was observed over the analysis period; however, during fully anaerobic conditions, both the DMK₈ and the MK₈ content are known to be high (Unden, 1988).

Effect of a shift to anaerobiosis in controlled carbon-limited chemostat conditions

Kinetics of MK₈ production

To determine the kinetics of MK₈ synthesis, analysis after a shift to anaerobiosis was performed over a longer time frame. For this, growth was performed aerobically in a glucose-limited chemostat at a dilution rate of 0.35 h⁻¹, allowing long sampling times upon a shift to anaerobiosis, without disturbance of the culture. Complete anaerobiosis was achieved by sparging nitrogen into the culture, resulting in a decrease of dissolved oxygen tension (dOT) values below the sensitivity of the oxygen electrode after 3 min. Samples for quinone analysis were quenched using a

Fig. 3. In *E. coli*, restricted oxygen availability induces a fast production of DMK₈. Samples were directly quenched in a methanol/HClO₄ solution to minimize alterations during the isolation procedure. The x-axis shows the time of sampling after a shift to low aeration. At 5 s, the amounts of DMK₈ and MK₈ were below the detection limit (1 nmol g⁻¹). White bars, DMK₈; black bars, MK₈. Error bars indicate SD.

rapid sampler, which is designed to quench cells within 0.5 s (Lange *et al.*, 2001). As can be seen from Fig. 4, the transition resulted in a rapid reduction of the UQ pool from 10 % to approximately 40 %, followed by a reoxidation to a level of about 25 %. DMK₈ synthesis occurred rapidly after the transition, at an initial rate quite similar to that observed in exponential non-limited cultures (Fig. 3). Synthesis started within 1 min after nitrogen sparging, whereas oxygen was not completely depleted at that time point (Fig. 4). As with the batch cultures, this illustrates that DMK₈ synthesis can take place in the presence of oxygen. Further, the rapid onset of synthesis suggests the presence of the synthesis pathway under fully aerobic conditions. This supports the conclusions of Shestopalov *et al.* (1997) who used an inhibitor of translation to show that the biosynthetic machinery is always present under aerobic conditions, and is activated under anaerobic conditions.

Whereas DMK synthesis was initiated within 10 s after the shift to anaerobiosis, and DMK levels reached a maximum within 1 min (Fig. 3), the increase in the total MK content was lower and much more gradual, with levels appearing to rise slowly, but continually, over the entire 15 min measurement period (Figs 3 and 4). Note that S-adenosyl-methionine : 2-demethylmenaquinone methyltransferase

(the *ubiE* gene product methylating DMK to MK) should be present under these conditions (Shestopalov *et al.*, 1997).

Redox state of the UQ pool during exponential growth

During exponential growth on glucose, the specific respiration rate of *E. coli* is relatively high (typically 8–10 mmol oxygen h⁻¹ (g dry weight)⁻¹; e.g. Calhoun *et al.*, 1993). Provided sufficient oxygen is available to the cells, under these conditions a (pseudo-)steady state is generally presumed to exist with respect to cellular composition, energy and redox state. In growth medium in which the stationary phase is brought about by exhaustion of the electron donor, one would expect the UQ pool to become largely oxidized upon entry of the cells into stationary phase. The rapid changes observed, and the high sensitivity of the respiratory chain with respect to the availability of oxygen, urged us to test this assumption. It was observed that in an experimental set-up that is representative of the common practice of closed exponential growth experiments, the percentage of reduced UQ₈ increased during the exponential phase of growth, suggesting that electron transfer to oxygen is impeded by the limited availability of oxygen (see Fig. 5). It is noteworthy that the increase occurred from OD₆₀₀ values as low as 0.3, which is equivalent to a biomass concentration of approximately 0.1 g dry weight l⁻¹, and at dissolved oxygen percentages as

Fig. 4. MK₈ and DMK₈ synthesis, percentage UQ₈H₂, and percentage oxygen saturation, upon a shift to anaerobiosis in controlled chemostat conditions. Samples were quenched using a fast sampler, which ensures that quenching times are below 0.5 s. The x-axis shows the time of sampling after a shift to anaerobiosis; *t*=0 is the time of shift to anaerobiosis. (A) ◆, UQ₈H₂; ●, oxygen saturation. (B) ▲, DMK₈; ■, MK₈; ◆, UQ₈. Error bars indicate SD.

high as 80 %. These dry weights are comparable to amounts used routinely to circumvent artefacts due to a high cell concentration (Constantinidou *et al.*, 2006). Interestingly, the increase in redox state of the UQ pool was mainly caused by a decrease in the oxidized UQ content (data not shown), whilst the reduced UQ content was stable throughout growth. The cellular UQ_8H_2 content was constant during exponential growth, but showed a decrease in the stationary phase. As shown in Fig. 5, true exponential growth was observed throughout the growth phase, with a constant doubling time of 0.9 ± 0.05 h. According to expectations, the UQ pool became more oxidized upon entry into stationary phase, which coincides with glucose depletion as measured routinely by HPLC (data not shown). Cells grown in nitrogen-limited medium showed similar temporal alterations in the redox state of the UQ pool (data not shown). These latter cells continue to oxidize glucose during the stationary phase, but at a much lower rate (see Discussion).

Quinone content during exponential growth

In a separate experiment, the amounts of the various quinone types were quantified during exponential growth. The data are shown in Fig. 6. UQ is known to be the most abundant quinone during aerobic growth of *E. coli*. Shestopalov *et al.* (1997) reported a UQ content of $300 \text{ nmol (g dry weight cells)}^{-1}$ during mid-exponential growth. The amounts measured in our experiments were up to almost 10-fold higher, particularly at the onset of exponential growth. It cannot be excluded that these differences are due to differences in growth medium (simple salts versus Luria broth) and/or the *E. coli* strain used (MC4100 versus ECL942), but it is noteworthy that the UQ content decreased continuously (to about 20 % of the initial content) throughout the exponential phase. In the earlier report by Shestopalov *et al.* (1997), cells were

Fig. 5. Growth-phase dependence of the redox state of the UQ_8 pool of *E. coli* during growth in minimal medium with glucose. The reduction state of the UQ_8 pool was measured in minimal medium supplied with 50 mM glucose in shake cultures. ■, UQ_8H_2 ; ●, oxygen saturation; ◆, OD_{600} . Data are from a single representative experiment.

Fig. 6. Biomass-specific quinone content during growth in minimal medium with glucose. The composition of the quinone pools was measured in minimal medium supplied with 50 mM glucose in shake cultures. ■, total UQ_8 (oxidized + reduced); ●, DMK_8 ; ▲, MK_8 ; ◆, OD_{600} . Error bars indicate SD.

collected by centrifugation before assessment of the quinone content. Together, these findings may well explain the observed differences. In agreement with Shestopalov *et al.* (1997), it was observed that DMK_8 was present under the conditions tested. Its presence showed a strong decrease upon entry into stationary phase.

DISCUSSION

Organisms like *E. coli* show an enormous metabolic flexibility that allows them to proliferate in many different environments, including with respect to carbon sources and the availability and nature of external electron acceptors, on the one hand, and to cope with sudden changes in many physicochemical parameters, such as nutrient concentrations, osmolarity, pH and temperature, on the other. The respiratory chain constitutes a major catabolic module that brings this flexibility about (Neijssel & Teixeira de Mattos, 1994). This is due first to the respiratory chain being branched, which allows for a variable efficiency of free energy conservation (Calhoun *et al.*, 1991, 1993; Calhoun & Gennis, 1993), and second to the potential to express a wide range of electron-transferring mediators and redox enzymes with different redox potentials and specificities, respectively (Berg & Stewart, 1990; Cole, 1996). Hence, a broad range of external electron acceptors can be used by the organism.

Quantitative studies on functional, regulatory and structural aspects of the respiratory chain demand well-defined and well-controlled experimental set-ups in which the availability of both electron donors and acceptors is either at a constant subsaturation level, or saturated throughout the experiment. Whereas the former condition can be obtained by the use of chemostats (Alexeeva *et al.*, 2000; Hommes *et al.*, 1991), the latter is usually presumed to occur in well-shaken batch cultures grown under standard conditions. It is common practice to consider a culture that shows exponential growth to be in a steady state with

respect to its macromolecular composition and enzymic make-up on the one hand, and with respect to nutrient availability on the other. We have shown, however, that this is not the case for *E. coli* grown under the standard batch conditions mentioned above. In order to quantify the regulation of the composition and redox state of the respiratory chain, a sensitive method was developed to determine the nature and content, as well as the redox state of the quinone pools. Optimization of the HPLC-based analysis methodology introduced by Shestopalov *et al.* (1997) and Uden (1988) resulted in sufficient resolution to discriminate the reduced and oxidized forms of UQ, MK and DMK at the nanomole per gram dry weight level. In addition, it is shown that the use of rapid sampling techniques to quench metabolism within a few seconds is paramount to obtain representative data because the redox state of the UQ changes significantly beyond this time window upon changes in stirring rate. In addition, the results presented here further illustrate the need for such rapid techniques even for cells that are not provided sufficient oxygen start to accumulate DMK₈.

Our analysis shows clearly that although exponential growth does occur in a batch culture that is aerated by orbital shaking, dramatic changes occur during this growth phase with respect to the redox state and the absolute content of UQ. Whereas, initially, in low-density cultures, the redox state of UQ was found to be the same as determined for highly aerated cultures, such as chemostat cultures (10 % UQH₂), or controlled batch cultures equipped with baffles and forced airflow (40 % UQH₂, data not shown), the UQ pool became more reduced as the exponential phase progressed, even at low culture densities (OD₆₀₀ ~0.2–1.0) and relatively high oxygen saturation (100 to ~80 %). In addition, with low-glucose batch cultures, the cellular content of the total pool of oxidized plus reduced species decreased. Concomitant with this decrease, DMK₈ and MK₈ were synthesized. The presence of DMK₈ in cultures that were presumably fully aerobic has also been reported by Shestopalov *et al.* (1997).

These results justify two important conclusions. First, upon oxygen restriction, *E. coli* responds on a second-scale response time with synthesis of quinones involved in anaerobic respiration. Second, the total oxygen consumption by the cells may rapidly exceed the oxygen transfer rate from the gas phase to the liquid phase in simple experimental setups such as Erlenmeyer flask cultures in an orbital shaker. This leads to growth conditions that are limited by the availability of oxygen, and therefore results in an increase in the degree of reduction of the components of the respiratory chain. Whether this is the case depends on both the respiratory activity of the cells and the cell density. This may explain the observation that, upon entry into the stationary phase, reoxidation of the UQ pool occurs: non-growing cells have a lower energetic demand and express a lower specific respiration rate (Teixeira de Mattos & Neijssel, 1997).

As mentioned above, the transition to conditions with low oxygen availability was accompanied by net synthesis of DMK₈. It has been shown that the biosynthesis machinery for MK₈ is present under aerobic conditions (Shestopalov *et al.*, 1997). The observed fast initiation of net synthesis of this compound suggests that its synthesis is not regulated at the transcriptional or translational level. The data indicate regulation at the post-translational level, i.e. it is determined by the ambient metabolite concentrations and the kinetics of the enzymes of the MK₈ biosynthetic pathway.

The technical and experimental difficulties of maintaining oxygen-saturated conditions in batch cultures have been addressed (e.g. Alexeeva *et al.*, 2000). The results presented in this paper describe semiquantitatively the important consequences of inadequate aeration on the physiology of *E. coli*, and the functioning of its respiratory machinery. They are of importance to research in the field of regulation of redox-related processes, whether they are at the molecular, genetic or physiological level. Currently, we are focusing on a fully quantitative description of the phenomena by making use of chemostat cultures, controlled batch cultures with high oxygen-transfer rates, and high-resolution dissolved-oxygen measurements.

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