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Photolabile ubiquinone analogues for identification and characterization of quinone binding sites in proteins

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ABSTRACT

Quinones are essential components in most cell and organelle bioenergetic processes both for direct electron and/or proton transfer reactions but also as means to regulate various bioenergetic processes by sensing cell redox states. To understand how quinones interact with proteins, it is important to have tools for identifying and characterizing quinone binding sites. In this work three different photo-reactive azidoquinones were synthesized, two of which are novel compounds, and the methods of synthesis was improved. The reactivity of the azidoquinones was first tested with model peptides, and the adducts formed were analyzed by mass spectrometry. The added mass detected was that of the respective azidoquinone minus N_2. Subsequently, the biological activity of the three azidoquinones was assessed, using three enzyme systems of different complexity, and the ability of the compounds to inactivate the enzymes upon illumination with long wavelength UV light was investigated. The soluble flavodoxin-like protein WrbA could only use two of the azidoquinones as substrates, whereas respiratory chain Complexes I and II could utilize all three compounds as electron acceptors. Complex II, purified in detergent, was very sensitive to illumination also in the absence of azidoquinones, making the 'therapeutic window' in that enzyme rather narrow. In membrane bound Complex I, only two of the compounds inactivated the enzyme, whereas illumination in the presence of the third compound left enzyme activity essentially unchanged. Since unspecific labeling should be equally effective for all the compounds, this demonstrates that the observed inactivation is indeed caused by specific labeling.

1. Introduction

Quinone binding sites are frequently found in proteins involved in the bioenergetic processes within cells and organelles. The one of the most well understood energy coupling mechanism, that of the bc_1 complex or Complex III, occurs entirely via quinone intermediates in the so called Q-cycle,[1,2] whereas other membrane-spanning enzymes such as NADH:quinone oxidoreductase (Complex I[3]), succinate:quinone oxidoreductase (Complex II[4]), quinol oxidase[5] and cytochrome *bd*-oxidase[6] contain quinone binding sites with more or less well characterized properties. Some membrane proteins have a purely regulatory quinone binding site like the sensor kinase RegB.[7] Soluble proteins such as flavodoxin[8] and membrane associated proteins like G3P dehydrogenase[9] also interact with quinone in the membrane, donating electrons to the quinone pool. Quinone-like inhibitors are important tools to better understand the functional mechanism of enzymes using quinone substrate, but they are also frequently used as insecticides, miticides, piscicides, and herbicides.[10–12] Exposure to such quinone-like inhibitors may cause free radical induced mutations to mitochondrial DNA that years later lead to the onset of neurodegenerative diseases like Parkinson's disease.[13] Some of the more serious side effects of anthracycline derived chemotherapeutical agents like adriamycin and doxorubicin are related to their ability to act as quinone binding site agonists or antagonists, causing free radical generation and cardiotoxicity.[14–16] At the same time, some quinone binding proteins are attractive putative drug targets.[17] As more high resolution structural information are becoming available for bioenergetic enzymes, and our understanding of the functional mechanisms increase concomitantly, there are still many cases where the exact location and architecture of quinone binding sites and/or inhibitor binding remains unidentified.

[^{3}H]azidoquinones have been successfully used in the past, to specifically label quinone binding sites by UV-illumination and subsequently detect and identify the radiolabeled protein. This technique, taking advantage of the dark stability and light sensitivity of photo probes, is becoming an increasingly common tool in

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drug discovery and development in general. A variety of azidoquinone compounds have been synthesized over the years, some showing good activities in some enzymatic systems but not in others. For example, in *Bos taurus* Complex II both membrane anchor polypeptides were photolabeled with [^{3}H]arylazidoquinone derivatives.[18] In subsequent labeling studies using the same enzyme, two peptide regions, one in SdhC and the other in SdhD, were assigned as quinone binding sites.[19,20] *Escherichia coli* Complex II was photo affinity labeled with 3-azido-2-methyl-5-methoxy-[^{3}H] 6-geranyl-1,4-benzoquinone ([^{3}H]azido-Q).[21] The Complex III quinone binding sites were labeled with 3-azido-2-methyl-5-methoxy-6-(3,7-dimethyloctyl)-1,4-benzoquinone.[22] Complex I from *Neurospora crassa* mitochondria was labeled with 2,3-dimethoxy-5-methyl-6-[7-(4-azido-3-[^{125}I] iodophenyl) heptanyl]-*p*-benzoquinone. This compound functioned as a substrate for Complex I, and upon illumination one polypeptide was labeled with high specificity.[23] However, the 9.5 kDa labeled polypeptide corresponded to a subunit not present in the bacterial Complex I, illustrating the risk of placing the azido group too distantly from the functionally conserved part of the molecule. Purified *E. coli* Complex I was recently labeled with 3-azido-2-methyl-5-methoxy [^{3}H]-6-decyl-1,4-benzoquinone, that was bound primarily to a peptide sequence in the NuoM subunit. Labeling of this site was however only slightly affected by five different Complex I inhibitors,[24] demonstrating that in an enzyme complex with more than one quinone binding site, a particular azidoquinone variant may specifically label only one of the sites. Direct labeling with various quinone site inhibitors is also common. Most recently an azido-labeled quinazoline-type inhibitor was crosslinked to the 49 kDa subunit of Complex I from bovine heart[25] whereas an acetogenin inhibitor was used to photo affinity label the ND1 subunit of the same enzyme.[26]

Taken together, the direct labeling of quinone binding sites is a fruitful strategy, but a much greater number of azidoquinone variants are needed, to be able to target and characterize all putative binding sites in quinone utilizing proteins. However, the previously employed methods for synthesis of azidoquinones have been hampered by low or very low yields, limiting the overall availability of the compounds to the scientific community.

A recent improvement to the original azidoquinone labeling strategy developed by Chang-An Yu,[18] is to detect the labeled peptides with mass spectrometry. Using this approach, the quinone analogues can be constructed with only one molecular modification, the azido group. The feasibility of this approach was recently demonstrated by Matsumoto et al.[6] in labeling the ubiquinol binding site of cytochrome *bd* from *E. coli*.

In this work we describe the construction of three different photo-reactive quinone analogues, using a safe and more effective synthesis strategy, and subsequent characterization of the azidoquinone reactivity using three different enzyme model systems; a soluble, single polypeptide protein, a membrane protein complex solubilized from the membrane with detergent, and a membrane protein complex in situ in the natural membrane. For effective labeling, the azidoquinone should closely resemble the natural substrate. The azidoquinone chosen must function as a substrate for the enzyme to be labeled, and ideally the activity should also exhibit normal inhibitor sensitivities. The labeling conditions should also be optimized for each target enzyme, finding the most effective illumination time and quinone:enzyme stoichiometry.

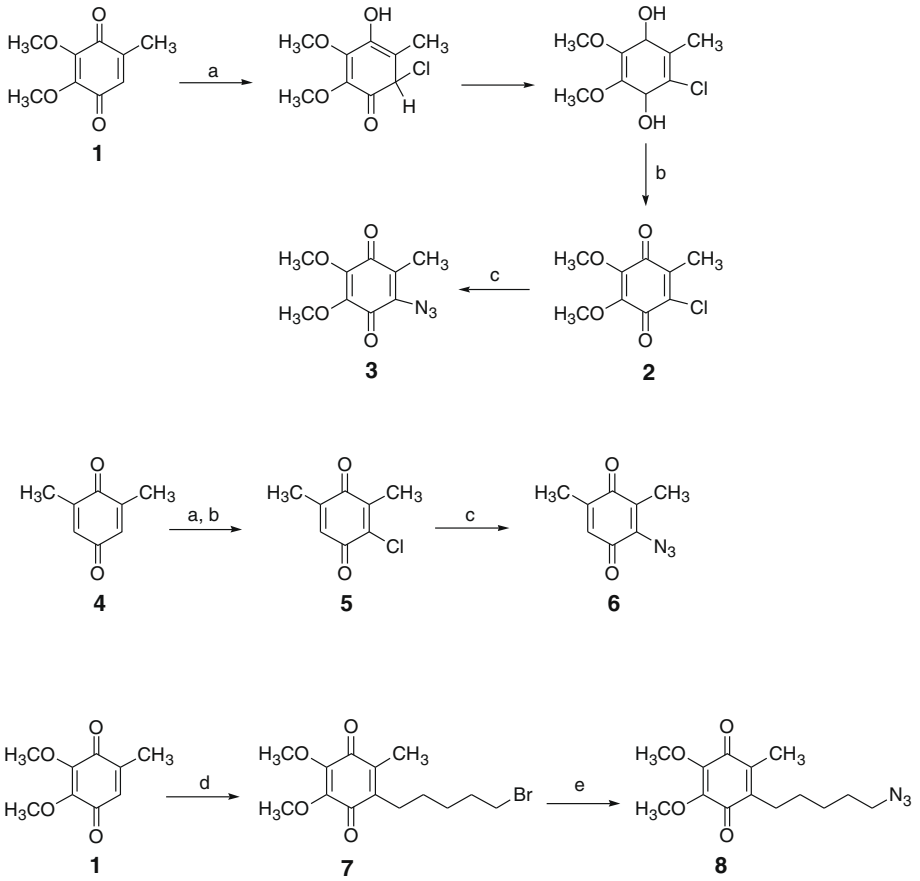

Figure 1. Synthesis of AzQ_0 (**3**), DAzB (**6**), and AzQ_1 (**8**), the two latter being novel compounds. AzQ_0 has been synthesized previously, by a less efficient method.[22,27] Reagents and conditions: (a) 37% HCl, H_2O, room temperature; (b) 67% HNO_3, Et_2O, 0 °C, 3 h, 10 °C, 1 h; (c) NaN_3, H_2O, 95% ethanol, room temperature, 1 h; (d) $(NH_4)_2S_2O_8$, $AgNO_3$, $Br(CH_2)_5COOH$, H_2O, CH_3CN, 65 °C, 1.5 h; (e) surfactant pillared clay, NaN_3, H_2O, $CHCl_3$, 95 °C, 3 h.

2. Results and discussion

2.1. Choice of quinones

The low solubility of the naturally occurring ubiquinone (from Q_6 to Q_{10}) in aqueous solution and the low absorption of quinones in the visible region has made studies of reaction mechanisms and protein:quinone interactions more difficult than other similar reactions. Low molecular weight quinone analogues, especially those with a reporting group, have offered a promising approach to study the protein:quinone interaction.[22,27] To be able to label all sites with affinity for the natural ubiquinone, it is desirable to put the azido group at a position where it is less likely to interfere with the biological activity. Given the results from the *N. crassa* Complex I labeling,[23] we also want to avoid having the reacting azido group located too far away from the quinone head. Therefore, we have chosen to synthesize ubiquinone derivatives with the azido group either located at a position on the benzoquinone ring, or at the end of a shorter aliphatic segment (Fig. 1). Since we will use mass spectrometry to identify the labeled peptides, we can utilize quinone analogues with only one substituent modification, the azido group.

2.2. Chemistry

A previously described method for the synthesis of 2,3-dimethoxy-5-azido-6-methyl-1,4-benzoquinone (AzQ_0) **3** from 2,3-dimethoxy-6-methyl-1,4-benzoquinone (Q_0) **1**, and compound **6** from **4**, was based on a direct replacement of a hydrogen atom on the benzoquinone ring with an azido group under weakly acidic conditions. The yield was very low (2%)[22,27] and the reaction was difficult to control, often resulting in large amounts of 2,3-dimethoxy-5-amino-6-methyl-1,4-benzoquinone being produced. An alternative strategy was devised by Miyoshi,[28] synthesizing 2-azido-3-methoxy-5-methyl-6-geranyl-1,4-benzoquinone (azido-ubiquinone-2) from a 2-hydroxy-3-methoxy quinone precursor. In that work the hydroxyl group on the ring was converted to a mesyl ester and the resulting compound was treated with NaN_3 to obtain the azidoquinone, with an overall yield of 24%.The method we instead used, was to react **1** (or **4**) with aqueous HCl to get an addition product (not isolated), which was subsequently oxidized by HNO_3 to obtain **2** (or **5**), followed by substitution of the chloride for the azide group resulting in **3** (or **6**). This reaction can be performed under mild conditions at 0 °C, and the overall yield was around 50%.[29]

To synthesize 2,3-dimethoxy-5-(5-azido-pentyl)-6-methyl-1,4-benzoquinone (AzQ_1) (**8**) using **1** as a starting material, we first produced 2,3-dimethoxy-5-(5-bromo-pentyl)-6-methyl-1,4-benzoquinone (Q_1-Br) (**7**) via radical alkylation of the quinone. The radical was generated from the decarboxylation of the corresponding bromo-acid with silver ion and peroxodisulfate.[30,31] This is a safer method than using $SOCl_2$ and H_2O_2 to produce the radical,[23,32] since the organic peroxides produced in the latter reaction decompose rapidly and may explode at high temperature. In the subsequent step, synthesizing **8** from **7** with sodium azide, it is particularly important to obtain complete conversion since **8** and **7** have similar polarities making separation difficult. We achieved a 93% yield using surfactant pillared clay as a phase transfer catalyst.[33,34]

2.3. Photo-decomposition of the azidoquinones

Photo-decomposition of AzQ_0 and DAzB can be conveniently monitored by optical spectroscopy and thus these compounds were used to optimize the experimental setup. Since the enzymes themselves typically also will be damaged by prolonged UV-irradiation it is important to find conditions were labeling occur but unspecific damage to the enzyme is minimized. Both azido-compounds reacted in a light-intensity, distance and time dependent fashion following first order kinetics (Fig. 2). DAzB decomposes somewhat faster than AzQ_0, but this may be explained by that the parent compound DMB (without azido group) is also more sensitive to UV-illumination than Q_0.

The products formed after illumination of AzQ_0 and DAzB were subjected to NMR and HRMS analyses. The former analyses indicated the presence of several different compounds that could not be assigned with certainty (not shown) whereas in the latter, products with mass corresponding to the azidoquinone minus N_2 can be assigned (See Supplementary data Fig. S1). When higher concentrations of azidoquinone were illuminated, gas bubbles were observed. For AzQ_0 the peak of 195.05 correspond to the elemental composition of $C_9H_9NO_4$ and the peak of 197.05 correspond to the elemental composition of $C_9H_{11}NO_4$, representing the amino-quinone. We tentatively assign the structure of the former product as corresponding to product 1 and 2 in[6] and compounds **3** and **4** in[35] (See Supplementary data Fig. S1). For DAzB the same corresponding products were seen (not shown).

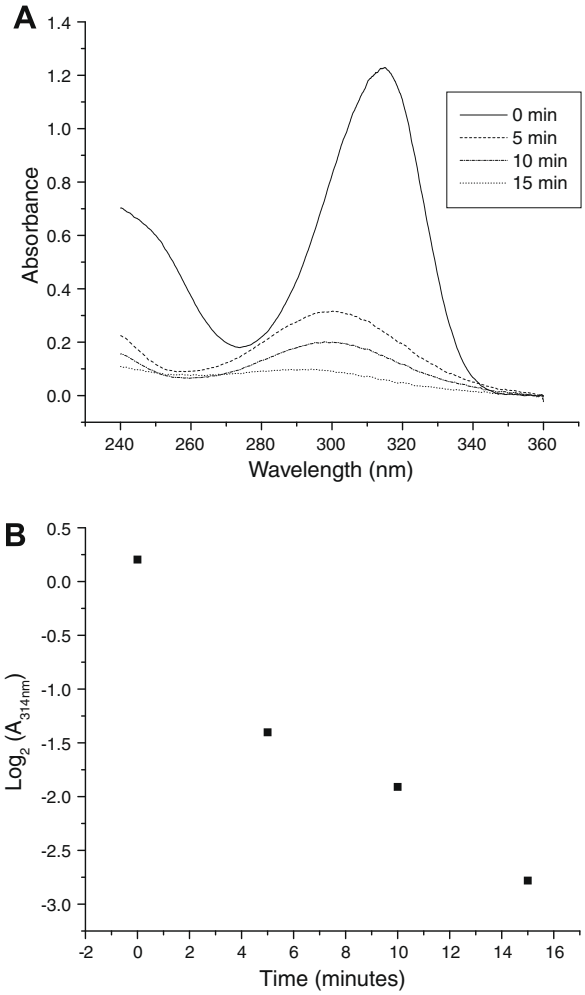

Figure 2. Bleaching of AzQ by UV-illumination. Optical spectra of AzQ_0 (compound **3**) during illumination (A) Photodecomposition of AzQ_0 over illumination time (B). 0.05 mM AzQ_0 in 95% ethanol was placed in a cuvette with 0.2 cm light path and mounted in an ice bath (0 °C). The sample was continuously illuminated with mid-wavelength UV light, and optical spectra were recorded with 5 min intervals. DAzB (compound **6**) reacted in an almost identical fashion (not shown).

2.4. Photolabeling of model peptides with azidoquinones

To investigate the azidoquinone reactivity with amino acids residues, high concentrations of azidoquinone was illuminated in the presence of model peptides, and analyzed the resulting products with mass spectrometry. For AzQ_0 labeled peptides we detected mass shifts of 195.05 ± 0.01 *m*/*z*. For AzQ_1 labeled peptides we observed 265.11 ± 0.04 *m*/*z* mass shift, and in two cases 248.08 ± 0.04 *m*/*z*. The twin peaks in the latter case most probably result from a de-amidation (Fig. 3). Peptides with two or in some case up to three AzQ adducts could be detected (Fig. 3A:2 and not shown). DAzB yielded mass shifts of 149.06 ± 0.03 (not shown).

Recently, Matsumoto et al.,[6] used a similar approach as us, but with a different azidoquinone, for labeling of the quinone binding site in cytochrome *bd*. In that work, a glutamate residue was specifically labeled. In theory, using high concentration of azidoquinone and small model peptides instead of protein with a distinct quinone binding site should yield completely unspecific and random labeling. This should permit an investigation of which amino acid adducts that can be formed. To determine which amino acids were preferentially targeted, AzQ_0 labeled Angiotensin II (with peptide sequence DRVYIHPF) peptide was subsequently subjected to MALDI-TOF MSMS to determine which amino acids that had adducts. The b-ion series showed that the label was predominantly positioned on the N-terminal amine, Asp or Arg. The y-ion series support this since y1–y5 were only detected for unmodified peptide while y7 and y8 were seen both with and without adduct (Fig. 4). This demonstrates that arginine was preferentially labeled in this peptide, presumably at one of the amines in the side chain. Aryl azides have previously been shown to react with amines.[35] We can not determine if the Asp or the N-terminus are also labeled, since the a1, b1, and c1 ions are not present in the spectrum. In addition, immonium ions formed by the His and Tyr could be seen both in the native and the labeled state (Fig. 4) indicating that the azido group can in fact react with a wide range of side groups. It should also be emphasized that many peaks are not assigned, reflecting the promiscuous reactivity of the azidoquinone. If instead a quinone binding site is labeled, the MSMS analyses will have a dramatically improved signal to noise ratio.[6]

2.5. Choice of biological material for quinone site labeling trials

To test the usefulness of three azidoquinones under different circumstances, three different model systems of increasing complexity were chosen. As an example of a small, single subunit, soluble protein interacting with quinone we have chosen WrbA, a tryptophane repressor binding protein in *E. coli*. WrbA uses FMN as the sole prosthetic group and belongs to the flavodoxin family,[36] and structurally and functionally resembles eukaryotic NAD(P)H:quinone oxidoreductases.[37] WrbA was previously shown to possess NADH:quinone reductase activity and operates via a ping–pong mechanism, that is, both NADH and quinone interact with the same active site.[38]

Respiratory chain Complex II from *Bacillus subtilis* is composed of three protein subunits, a flavoprotein (FP) and an iron–sulfur protein (IP) protruding from the membrane, and a membrane

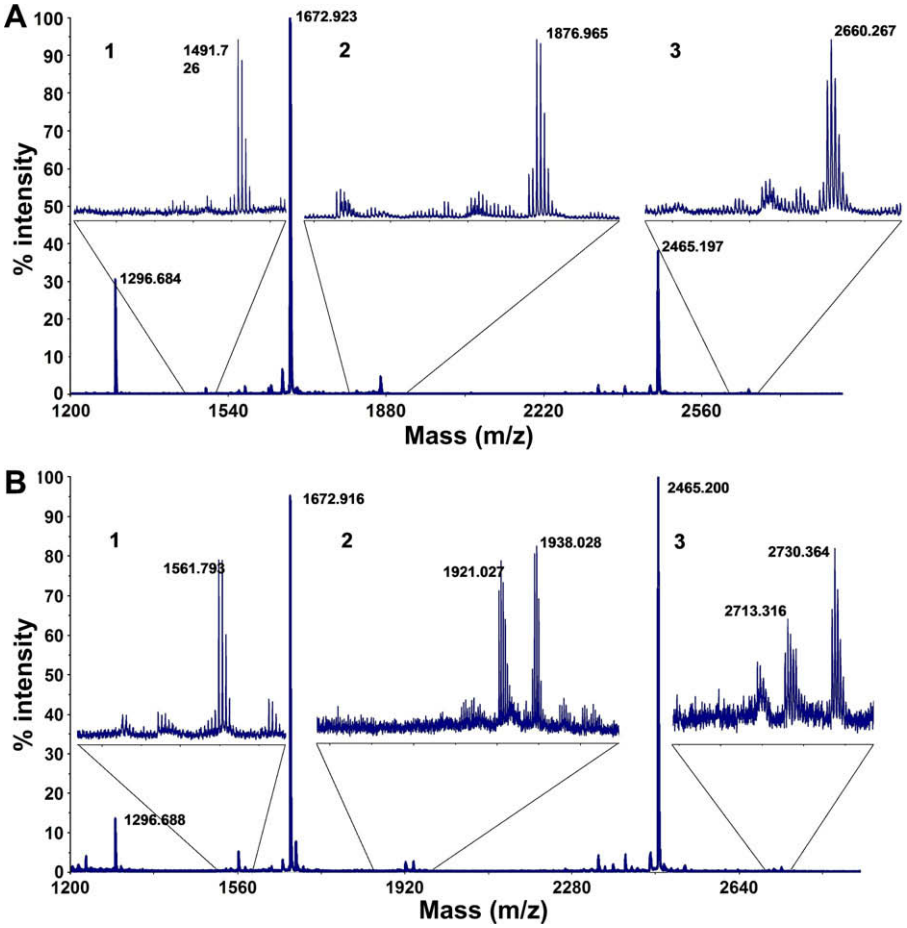

Figure 3. (A) Model peptides Angiotensin I (1, with amino acid sequence DRVYIHPFHL), Neurotensin (2, with amino acid sequence pELYENKPRRPYIL), and ACTH (18-39) (3, with amino acid sequence RPVKVYPNGAEDAEAFPLEF) illuminated in presence of AzQ_0 and (B) the same peptides illuminated in presence of AzQ_1. The peaks corresponding to peptide with AzQ adducts are enlarged in A1, A2, A3 and B1, B2, B3. The mass shift seen for the AzQ_0 labeled peptides in A is 195.05 ± 0.01 *m*/*z* and the mass shift seen for AzQ_1 labeled peptides in B is 265.11 ± 0.04 *m*/*z* or in two cases 248.08 ± 0.04 *m*/*z*. The twin peaks in the latter cases most probably result from a de-amidation.

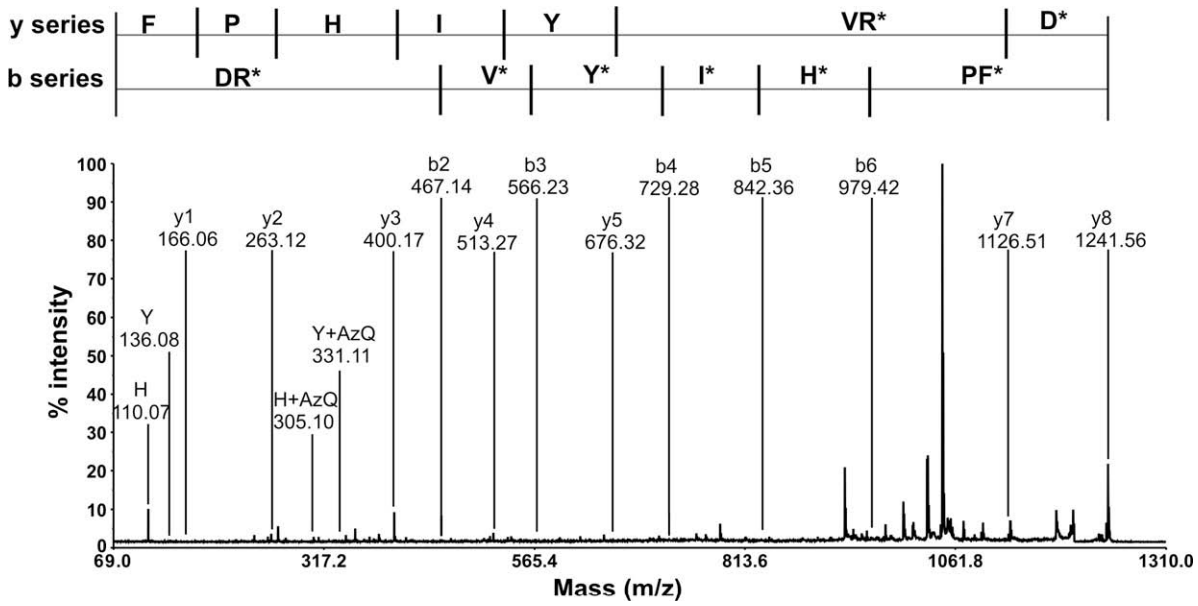

Figure 4. AzQ_0 labeled Angiotensin II (with amino acid sequence DRVYIHPF) subjected to MALDI-TOF MSMS. The star indicate mass of fragments with AzQ_0 adduct. The b-ion series show that the azidoquinone label was predominantly positioned on the aspartate or arginine. The y-ion series also support this since y1–y5 was only detected for unmodified peptide while y7 and y8 was seen both with and without the adduct. In addition, immonium ions formed by the His and Tyr could be seen both in the native and the labeled state. The dominant peak in the spectra at 1046.5 corresponds to the peptide that has lost the AzQ_0 adduct.

anchor cytochrome *b*. The FP contains a covalently bound FAD, the IP harbors three iron–sulfur clusters, and the cytochrome has two heme groups of *b*-type.[39] In vitro, the enzyme is able to catalyze both succinate:quinone reductase and quinol:fumarate reductase, depending on the midpoint potential of the added quinone.[40] Most likely, the enzyme contains two quinone binding sites, the Q_p site located at the interface between IP and the membrane anchor cytochrome, and the Q_d site located in the distal end of the membrane anchor polypeptide.[4] The specific inhibitor 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) blocks 90% or the enzyme, and is known to bind at the Q_d site. The Complex II enzyme was used since it is membrane spanning, but remains stable and active during purification in detergent solubilized state.[39]

Respiratory chain Complex I from *Rhodobacter capsulatus* is a large enzyme complex composed of 14 protein subunits, containing FMN and eight iron–sulfur clusters as prosthetic groups.[41] Seven subunits are membrane spanning and seven subunits form a large domain protruding from the membrane. The enzyme catalyses the NADH:quinone oxidoreductase reaction coupled to proton translocation across the membrane by an hitherto unknown mechanism.[42] Several quinone binding sites are presumably present in Complex I[43] and a number of specific inhibitors, for example rotenone and piericidin A, interfere with quinone binding.[44] However, the Complex I enzyme is notoriously unstable, and generally looses its ability to reduce quinones immediately upon addition of detergent.[45] Thus, Complex I was chosen to investigate the feasibility and effectiveness of azidoquinone labeling in situ, in a natural membrane. This experiment thus offers additional challenges in term of illumination efficiency in a turbid sample, and the presence of a protein mixture in the sample.

2.6. Biological activity and labeling efficiency of the azidoquinones in the chosen model systems

For effective labeling of quinone binding sites in proteins, it is important to use an azidoquinone that functions as substrate, preferably resembling the natural substrate as closely as possible. Since the natural ubiquinones are very hydrophobic and thus inconvenient to use in in vitro assays, the corresponding water soluble quinone analogues are used. In the test assays we have utilized the soluble quinones Q_0, DMB and Q_1 that are commercially available, and Q_0 and DMB were used as starting materials for synthesis of AzQ_0, AzQ_1 and DAzB.

2.6.1. WrbA

The activity of purified WrbA protein[46] was measured with the azidoquinones as substrates (Table 1) and the activities were compared to that using the structurally equivalent unmodified quinone analogues. For AzQ_0 and DAzB the activities are virtually the same as for Q_0 and DMB, demonstrating that the azido group did not interfere with the enzymatic function. The proposed physiological function of WrbA is to keep the quinone pool reduced under stress conditions,[38] but in fact WrbA has only been demonstrated to react with soluble quinones. Already when one isoprenoid unit was present, as in Q_1, the activity was almost completely abolished ([37], Table 1). Thus, it is not surprising that AzQ_1 did not work as a substrate for WrbA.

Subsequently, the WrbA protein was illuminated in the presence of AzQ_0 and DAzB for different time periods, after which the enzyme activity was measured as before. The azidoquinone content and illumination time was optimized, with best results obtained at about 5 times molar excess of azidoquinone. The best illumination time dependent inactivation of the enzyme is shown in Figure 5. Both azidoquinones inactivated the enzyme, but AzQ_0 was somewhat more effective than DAzB in blocking enzyme

Table 1
WrbA enzyme activity using the different quinone analogues as substrate

	V_{max} (μM/min × mg)
Q_0	684
AzQ_0	724
Q_1	<10
AzQ_1	nd
DMB	546
DAzB	464

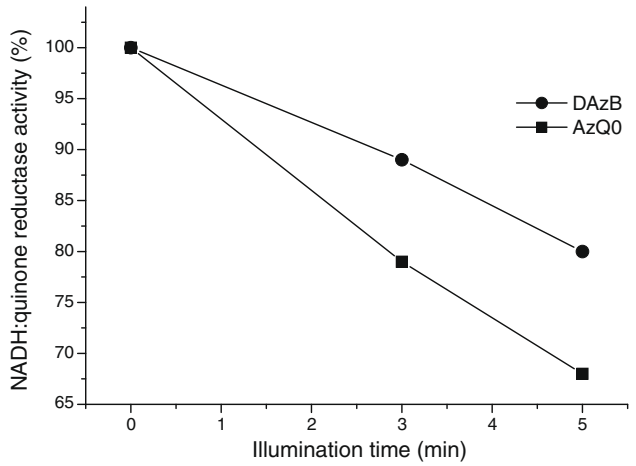

Figure 5. The NADH:quinone reductase activity of WrbA after photo affinity labeling with AzQ_0 or DAzB. Illuminated samples were collected and kept on ice in the dark. When the complete sample series had been collected, the activity was measured immediately. Unspecific inactivation, caused by illumination of sample in the absence of azidoquinone, has been deducted from the data presented above. Inactivation in the absence of azidoquinone was less that 5% during 5 min illumination.

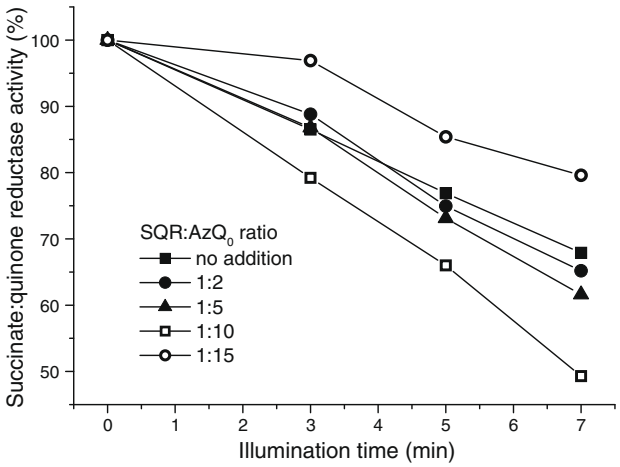

Figure 6. Succinate:quinone reductase activity after photo affinity labeling of detergent solubilized Complex II purified from *B. subtilis* using AzQ_0. Inactivation in the absence of azidoquinone, and in the presence of elevated azidoquinone concentrations are shown to illustrate the narrow window available to monitor the labeling process in this enzyme. After illumination, the samples where kept on ice in the dark until the complete sample series had been collected, and then the activity was measured immediately.

activity. In WrbA, inactivation during illumination in the absence of azidoquinone was less than 5% during 5 min of constant illumination, probably since photo-damaged FMN can be exchanged, and thus replenished, in this enzyme assay (see Section 3.5.1).

2.6.2. Complex II

The enzyme activity of Complex II was measured, both directly in the membrane-bound state and using the enzyme purified in detergent, as described in Section 3.4.1. All three azidoquinones were good substrates for the enzyme, and in the case of the most water soluble compound AzQ_0 and DAzB, the V_{max} was even a bit higher than for the unmodified corresponding quinone (Table 2). Furthermore, the reactions showed normal inhibitor sensitivity, with about 10% residual activity seem in the presence of the specific inhibitor HQNO. We then proceeded to determine the best labeling conditions for Complex II, in the same way as previously done for WrbA. The purified, detergent solubilized enzyme was mixed with different molar ratios of azidoquinone, and the sample was illuminated. Complex II turned out to be very sensitive to illumination also in the absence of azidoquinone (see Fig. 6). After 5 min of illumination, 25% of the Q_1 reductase activity is already lost, making it cumbersome to observe specific, azidoquinone-induced inactivation, even if specific labeling of the protein may still occur. Both succinate:quinone reductase activity and succinate reductase activity to the artificial electron acceptor phenazine methosulfate (PMS) was affected by illumination, but the latter activity to a much smaller degree (about 5% decrease after 5 min of illumination). Succinate:PMS reductase activity is however not HQNO sensitive,[39] and thus this activity does not involve the quinone binding sites, but must be bypassing part of the natural electron transfer pathway. Notably, when we attempted to compensate for the high unspecific light-dependent inactivation by adding higher molar ratios of azidoquinone, the inactivation efficiency instead decreases (Fig. 6). At concentrations above 50 μM azidoquinone the quinones start to act as a UV filter, illustrating the importance of carefully optimizing the labeling conditions. Incubation of enzyme with the photo affinity substrates in darkness did not result in any inhibition (not shown).

Table 2
Succinate:quinone oxidoreductase enzyme activity and inhibitor sensitivity using the different quinone analogues as substrate

	V_{max} (μM/min × mg)	I_{max} (% inhibition) HQNO
Q_0	2.19	88%
AzQ_0	3.72	89%
Q_1	2.52	90%
AzQ_1	2.57	nd
DMB	3.99	nd
DAzB	4.72	nd

2.6.3. Complex I

In Complex I in *R. capsulatus* membranes, all the soluble quinone analogues DMB, Q_0 and Q_1 were found to be reasonably good substrates (Table 3). The reactions were sensitive to classical Complex I specific inhibitors, although the maximal inhibition levels are somewhat lower than for the natural ubiquinone. The azidoquinones were subsequently evaluated for their ability to act as electron acceptors from respiratory Complex I during NADH oxidation, using. DMB, Q_0 and Q_1 as reference compounds as before. As

Table 3
NADH:quinone oxidoreductase enzyme activity and inhibitor sensitivity using the different quinone analogues as substrate

	App. K_m (μM)	V_{max} (μmol NADH/(min × mg))
Q_0	75	0,37
AzQ_0	52	0,58
Q_1	23	0,67
AzQ_1	21	0,35
DMB	100	0,35
DAzB	86	0,59
	I_{max} (% inhibition)	
	Piericidin A	Rotenone
Q_0	68	48
AzQ_0	68	53
Q_1	73	68
AzQ_1	79	79
DMB	75	63
DAzB	71	53

The maximal inhibition of the NADH oxidase reaction (i.e., using the natural ubiquinone in the membrane as electron acceptor) was 92% when using rotenone and 96% when using piericidin A. For experimental details, see Section 3.

seen in Table 3, the azido modifications did not hamper the interaction with the enzyme. Just as was seen for Complex II, the azido modified quinones DAzB and AzQ_0 are even better substrates than the unmodified quinone variants, as demonstrated by the increase in V_{max} and decrease of apparent K_m. For AzQ_1 there is a 52% decrease in V_{max} but about the same apparent K_m compared to Q_1. However, it must be noted that these two compounds do not only differ with respect to the azido group, since the isoprenoid chain in Q_1 is substituted for a saturated pentyl group in AzQ_1 (Fig. 1). In bovine Complex I it has been demonstrated that the position of the methyl group in the isoprenoid tail region of the quinone is recognized by the enzyme.[47] The structural difference might also influence the partitioning to the hydrophobic phospholipid bilayer and therefore the local concentration of the substrate at the binding site. Piericidin A and rotenone are both classical Complex I inhibitors, acting at the level of quinone reduction, but there is no consensus on if these inhibitors bind at the same location or if they target different, distinct quinone binding sites.[44] Even so, the sensitivity to both inhibitors of all the azido compounds are similar to those of the unmodified quinones (Table 3). Taken together we conclude that all three azidoquinone analogues accept electrons from the oxidation of NADH by Complex I in a similar way to the unmodified ubiquinone analogues. Since the Complex I enzyme is sensitive to several types of inhibitor compounds we also tested if any of the products formed from the azido quinones after illumination were Complex I inhibitors. *R. capsulatus* membranes were incubated with previously illuminated azidoquinone and the NADH oxidase activity was measured. The presence of 50 μM post-illuminated DAzB had no effect on enzyme activity whereas the same amount of photo-reacted AzQ_0 or AzQ_1 cause a 20% increase in activity. When the same amount of Q_0 or Q_1 was added, up to 30% maximal stimulation of activity was seen. This slight difference most likely reflect the amount of redox competent quinone remaining in the sample, but in any case we can rule out that any Complex I inhibitor was formed.

Then, we proceeded with labeling of the *R. capsulatus* Complex I in membrane vesicles. The experimental setup was optimized as before, to minimize unspecific radiation damage of Complex I and maximize specific labeling in the turbid bacterial membrane vesicles solution. Since the other respiratory chain enzymes also may become labeled, and we want to evaluate the effectiveness of Complex I labeling only, the enzyme activity after illumination was measured as NADH:Q_1 reductase activity rather than NADH:oxidase activity (see also Section 3). Keeping the sample on ice during illumination and using a light source to sample distance of 7 cm we obtained the best results. Under these conditions the loss of NADH:Q_1 reductase activity in a sample without added azidoquinone was less than 10% after 4 min illumination. At this time most of the azidoquinone in the sample had reacted. As a control we also monitored the NADH:ferricyanide reductase activity in the samples. It is not known if ferricyanide accepts electrons directly from FMN or from one of the iron–sulfur clusters, but since the NADH:ferricyanide reductase activity of Complex I is insensitive to rotenone or piericidin A, it is comparable to the previously described succinate:PMS reductase activity of Complex II, in that it does not involve the quinone binding sites. The NADH:ferricyanide reductase activity of the Complex I samples remained constant for up to 6 min illumination, regardless of if azidoquinone was present or absent. A pronounced decrease of NADH:ferricyanide reductase activity does not occur until after more than 20 min illumination (not shown). This indicates that a particularly UV sensitive functional group, perhaps the unidentified component described in,[48] seems to be present between the site of ferricyanide reduction and the site of quinone pool reduction. Incubation of enzyme with the photo affinity substrates in darkness showed no inhibition of NADH:Q_1 reductase activity. As seen in Figure 7, both AzQ_0 and

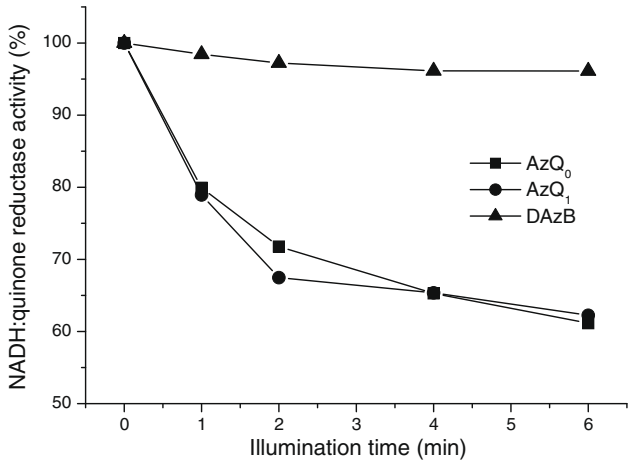

Figure 7. NADH:Q_1 reductase activity after photo affinity labeling of Complex I in *R. capsulatus* membrane vesicles with AzQ_0, AzQ_1, and DAzB. Activity was measured immediately after illumination of each individual sample. Unspecific inactivation, caused by illumination of sample in the absence of azidoquinone, has been deducted from the data presented above. The NADH:ferricyanide activity of Complex I did not change during the illumination time interval shown above, neither in the presence nor in the absence of azidoquinone.

AzQ_1 caused an illumination time dependent inactivation of Complex I NADH:Q_1 reductase activity but not of NADH:ferricyanide reductase activity (not shown), demonstrating that a specific photolabeling of the enzyme had occurred. We compared the photolabeling efficiency at azidoquinone concentrations ranging from 2 μM up to 100 μM. The labeling efficiency was slightly higher at 20 μM azidoquinone, (corresponding to the apparent K_m for Q_1 and AzQ_1 (Table 3)), but began to decrease at higher azidoquinone concentrations. At higher concentrations the excess azidoquinone again acted as a UV filter, actually reducing the amount of light reaching the enzyme and producing specific labeling. To reduce the amount of unspecific labeling of the enzyme, it is also preferred to keep the concentration of azidoquinone low. The data shown in Figure 7 were obtained using 20 μM of each of the three azidoquinones. In *N. crassa* Complex I, the azidoquinone labeling efficiency was increased in the presence of NADH.[23] In our experiment, no difference in labeling efficiency was seen when the enzyme was preincubated with 5 μM NADH (in the presence of KCN) and subsequently illuminated. Addition of 5 μM NADH together with 20 μM of the azidoquinone electron acceptor immediately before illumination also did not affect the labeling behavior (not shown).

Interestingly, DAzB, that functioned well as a substrate for Complex I (Table 3) and showed even higher light sensitivity than AzQ_0, (not shown) did not inactivate the enzyme. The sample illuminated in the presence of DAzB (Fig. 7) behaved essentially like a sample without added azidoquinone. DAzB is indeed the compound that structurally deviate the most from the natural ubiquinone since the two methoxy groups in ubiquinone are replaced by a single methyl in DAzB (Fig. 1 and Table 3). The failure of DAzB to act as a specific labeling agent must reflect specific features in the architecture of the binding sites, but also further strengthens the notion that the other two compounds have indeed targeted the natural quinone binding sites and labeled the enzyme specifically.

2.7. Conclusion

In this work we have synthesized three azidoquinone analogues, two of which are novel compounds, and tested their ability to target and specifically label quinone binding sites in different types of enzymes. All three compounds reacted with model peptides upon illumination, causing mass shifts in the peptides corresponding to the mass of the respective azidoquinone minus N_2 gas.

Light-dependent inactivation in the presence of azidoquinones could be seen both using purified, water soluble (Fig. 5) or detergent solubilized enzyme (Fig. 6) and in a turbid membrane vesicle solution, where the azido-compounds had to compete for the binding sites with the natural ubiquinone (Fig. 7). In WrbA, only the two most hydrophilic quinones could be used as substrates, whereas neither Q_1 nor the corresponding AzQ_1 showed any enzyme activity (Table 1). In Complex II, all three azidoquinones were as good substrates or better as the corresponding quinone without the azido group (Table 2), but the labeling efficiency was difficult to estimate accurately, due to the high degree of UV-induced inactivation of the enzyme also in the absence of azidoquinone (Fig. 6). Future mass spectrometric analyses will have to reveal the de facto efficiency of labeling in these samples. In membrane bound Complex I, two of the compounds, AzQ_0 and AzQ_1, fulfilled the criteria of being accepted as substrates, showed normal inhibitor sensitivity and reacted as specific photo-affinity labels. The third compound, DAzB, fulfilled the first three criteria but failed to produce light-dependent inactivation of Complex I (Table 3, Fig. 7). Since the three compounds are chemically similar but structurally different this finding further strengthens the notion that labeling, specifically targeting the quinone binding sites have occurred in the other two cases.

3. Experimental section

3.1. Chemistry

All chemicals used were of reagent grade. Reactions were routinely monitored by thin layer chromatography (TLC) on silica gel plate (F_{254} Merk), and the products were visualized with UV lamp. Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. ^1H and ^{13}C spectra were recorded with a Bruker Avance 400 instrument or a Bruker DMX 500 instrument at 298 K in $CDCl_3$, using the residual signals from $CHCl_3$ (^1H: δ = 7.27 ppm; ^{13}C: δ = 77.0 ppm) as internal standard. The infrared spectra (IR) were recorded on a Shimadzu FT-IR-8300 instrument. Flash chromatographies were performed using Merk Silica Gel 60 Å. Starting materials 2,3-dimethoxy-6-methyl-1,4-benzoquinone (Q_0) and 2,6-dimethyl-1,4-benzoquinone (DMB) were purchased from Sigma–Aldrich.

3.1.1. 2,3-Dimethoxy-5-chloride-6-methyl-1,4-benzoquinone (Q_0-Cl) (2)

1.5 mL of 37% hydrochloric acid was diluted with 0.5 mL of H_2O mixed with **1** (1.35 mmol, 245 mg). The mixture was stirred for 3 h at room temperature, then poured onto 5 mL of ice-cold water and extracted with diethyl ether. The ether phase was washed with a saturated sodium chloride aqueous solution until it was neutral, and then the solvent was removed under reduced pressure. The residue was taken up with 5 mL ether, cooled to 0 °C and treated with 0.18 mL HNO_3 for 3 h, then stirred for 1 h at 10 °C. Then the mixture was poured onto 5 mL of ice-cold water and extracted with diethyl ether. The ether phase was washed with a saturated sodium chloride solution, dried over anhydrous $MgSO_4$, and then filtered. The solvent was removed under reduced pressure to get the orange–red solid. The crude product was purified by recrystallization from cyclohexane to give product **2** (yield 62%). Melting point 65–67 °C.[30] ^1H NMR ($CDCl_3$) δ 2.02 (s, 3H, CH_3), 4.02 (s, 6H, OCH_3). IR 2955 cm^{-1} (C–H), 1649 cm^{-1} (C═O), 1610 cm^{-1} (C═C).

3.1.2. 2,3-Dimethoxy-5-azido-6-methyl-1,4-benzoquinone (AzQ_0) (3)

A solution containing 60 mg (0.925 mmol) sodium azide in 0.75 mL water was added to a solution containing 80 mg (0.462 mmol) **2** in 95% ethanol (2 mL), and stirred for 1 h at room temperature. The reaction mixture was poured onto 7.5 mL of ice-cold water and extracted with ethyl ether, then dried over anhydrous $MgSO_4$, and filtered. The solvent was removed under reduced pressure to leave the crude product. Compound **3** (yield 80.0%) was obtained after recrystallization with cyclohexane. Melting point 49–51 °C (*27*). ^1H NMR ($CDCl_3$) δ 2.02 (s,3H,CH_3), 4.00 (s, 6H,OCH_3). IR 2955 cm^{-1} (C–H), 1649 cm^{-1} (C═O), 1610 cm^{-1} (C═C), 2130 cm^{-1} (–N_3).

3.1.3. 2,6-Methyl-5-azido-1,4-benzoquinone (DAzB) (6)

Compound **6** was synthesized by using the same method as for the synthesis of **3**. The final yield was 20%. ^1H NMR ($CDCl_3$) δ 1.98 (s, 3H, CH_3-2), 2.10 (s, 3H, CH_3-6), 6.58 (s, 1H, Ar-H); ^{13}C NMR ($CDCl_3$) δ 10.9, 16.0, 128.0, 131.1, 139.6, 147.3, 182.3, 186.2; IR 2137 cm^{-1} (–N_3), 1651 cm^{-1} (C═O), 1617 cm^{-1} (C═C); HRMS (FAB)) calcd for $C_8H_7O_2N_3$ [M+H]: 177.0538; found: 177.0558.

3.1.4. 2,3-Dimethoxy-5-(5-bromo-pentyl)-6-methyl-1,4-benzoquinone (Q_1-Br) (7)

A solution of 513 mg (2.25 mmol) $(NH_4)_2S_2O_8$ in 2.5 mL water was added drop wise over 1 h to a stirred suspension of 6 mL CH_3CN and 6 mL water with 273 mg (1.5 mmol) **1**, 150 mg $AgNO_3$ and 439 mg (2.25 mmol) bromo-hexanoic acid at 60–65 °C. After stirring for 20 min, the mixture was cooled to room temperature and extracted with diethyl ether, then washed with 1 M $NaHCO_3$. The ether phase was dried over anhydrous $MgSO_4$ and filtered, then the solvent was removed in vacuo at room temperature. Compound **7** (yield 10%) was obtained after flash chromatography using pentane/diethyl ether (2:1) as mobile phase. ^1H NMR ($CDCl_3$) δ 1.35–1.55 (m, 4H, CH_2–CH_2), 1.85–1.94 (m, 2H, CH_2–(CH_2–Br)), 2.03 (s, 3H, Ar-CH_3), 2.48 (t, 2H, J = 7.6 Hz, Ar-CH_2), 3.42 (t, 2H, J = 6.7 Hz, CH_2–Br), 3.99, 4.00 (s, 6H, 2 × OCH_3). IR; 2938 cm^{-1} (C–H), 1647 cm^{-1} (C═O) 1610 cm^{-1} (C═C), 1262 cm^{-1} (–CH_2–).

3.1.5. 2,3-Dimethoxy-5-(5-azido-pentyl)-6-methyl-1,4-benzoquinone (AzQ_1) (8)

Montmorillonite (5 g) was first stirred with tetrabutylammonium bromide (3.56 g) in 50 mL water for 120 h at 60–70 °C. The solution was filtered, and the clay was washed repeatedly with distilled water and dried in an oven at 100–110 °C over night.[34] Compound **7** (16.5 mg, 0.05 mmol) in chloroform (0.5 mL), and sodium azide (3.9 mg, 0.06 mol) in water (0.5 mL) were mixed in a round-bottomed flask. The surfactant pillared clay (5 mg) was added, then the reaction mixture was refluxed with constant stirring for 3 h at 90–100 °C until all the starting material was consumed. The reaction was quenched with water and the product extracted with chloroform (2 × 1 mL). The combined extracts were washed three times with water, dried over anhydrous $MgSO_4$ and filtered. The solvent was removed under reduced pressure, to obtain **8**, an orange oil. The yield was 93%. ^1H NMR ($CDCl_3$) δ 1.40–1.48 (m, 4H, CH_2–CH_2), 1.59–1.68 (m, 2H, CH_2-(CH_2–N_3)), 2.03 (s, 3H, Ar-CH_3), 2.48 (t, 2H, J = 6.9 Hz, Ar-CH_2), 3.29 (t, 2H, J = 6.8 Hz, CH_2–N_3), 3.99, 4.00 (s, 6H, 2 × OCH_3). IR 2940 cm^{-1} (C–H), 2096 cm^{-1} (–N_3), 1648 cm^{-1} (C═O), 1610 cm^{-1} (C═C), 1266 cm^{-1} (–CH_2–). ^{13}C NMR ($CDCl_3$) δ 11.9, 26.2, 26.9, 28.2, 28.6, 51.3, 61.2, 138.9, 142.5, 144.3, 184.1, 184.6; HRMS (FAB) calcd for $C_{14}H_{19}N_3O_4$ [M+H]: 294.1454; found: 294.1451.

3.2. Photodecomposition of azidoquinones

DAzQ and AzQ_0 were diluted to 500 μM in 15 mL mH_2O and illuminated with mid-wavelength UV light for up to 10 min at a 7 cm distance from the light source (UVM-57 (UVP Inc.)). The products formed were collected after evaporating the mH_2O and dissolved in D_2O prior to NMR analysis or dissolved in mH_2O prior

to HRMS analysis, performed on a GCT EI+ (Micromass). When monitoring the photodecomposition by optical spectroscopy DAzQ or AzQ_0 was dissolved to 50 μM in ethanol and illuminated with the same light source directly in the cuvette.

3.3. Photolabeling and MS analysis of model peptides

Four different commercially available model peptides, Angiotensin I, Angiotensin II, Neurotensin and ACTH (18-39), were mixed with a 200 mol excess of the different AzQs. The mixture was illuminated for 4 min on a hydrophobic film placed on ice, at the same distance from the light source as before. The peptides were dried in darkness at room temperature, were redissolved in 0.1% TFA, desalted and spotted on to a MALDI target plate using Stage Tips as described in.[49] MALDI-TOF MS spectra of the peptides were collected using a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, CA) in reflector mode. Saturated α-cyano-4-hydroxycinnamic acid in 70% acetonitrile and 0.1% TFA was used as matrix. The spectra were calibrated internally using the known masses of the model peptides. MSMS spectra were collected in an automated mode with no collision gas present. The spectra were analyzed using Data Explorer 4.5 (Applied Biosystems) followed by a manual interpretation.

3.4. Biological material

WrbA from *E. coli*, prepared as in,[46] was a provided by professor Jannette Carey. The BCA Protein Assay Kit (Pierce) was used to determine protein concentrations. The Complex II concentration was determined spectroscopically using $\varepsilon_{558-575} = 45\ mM^{-1}$ of the dithionite reduced enzyme.[50]

3.4.1. Preparation of *B. subtilis* membranes and purified enzyme for Complex II labeling

B. subtilis 3G18/pBSD1200, that overproduce Complex II 3–4 times compared to wild type cells, was grown aerobically in NSMP medium containing 5 μg/mL chloramphenicol at 37 °C and harvested in the early stationary growth phase. The cytoplasmic membrane containing Complex II was prepared from the bacteria as previously described.[39] To purify Complex II, the membranes were solubilized in dodecylmaltoside (DDM) in 20 mM MOPS at pH 7.4 over night under gentle stirring, using 5 mg of detergent per mg of membrane protein. Unsolubilized material was removed by centrifugation for 1 h at 48,000*g*. The supernatant was applied on a DEAE-Sephacel column that had been pre-equilibrated with 20 mM MOPS buffer containing 0.1% (w/v) Thesit. Subsequent purification steps were done as previously described.[39] Peak fractions from ion exchange chromatography were pooled and concentrated using Centriprep spin columns with a cut off at 30 kDa. The sample was diluted in 20 mM MOPS, followed by repeated concentration to reduce the salt concentration. The concentrated sample was loaded on a S-400 gel filtration column pre-equilibrated with the MOPS buffer containing 100 mM KCl and 0.1% Thesit. A_{412} (the cytochrome *b* Soret band) and at A_{280} was measured on the eluted fractions. The purity and SQR content of the fractions was also estimated from SDS–PAGE gels, before pooling and concentrating the peak fractions using a Centricon-30 micro concentrator. All purification steps were performed at 4 °C and the purified enzyme was stored on ice in the dark. The Complex II concentration was determined spectroscopically using $\varepsilon_{558-575} = 45\ mM^{-1}$ and $\varepsilon_{428-412} = 269\ mM^{-1}$ of the dithionite reduced enzyme.

3.4.2. Preparation of *R. capsulatus* membranes for Complex I labeling

R. capsulatus (ATCC 17015) was grown aerobically in the dark at 32 °C in baffled E-flasks, in a 1:1 mixture of RCVBN (minimal medium) and MPYE (complex medium[51]) resulting in expression of high amounts of Complex I. The cells were harvested by centrifugation after reaching the late exponential growth phase. The cells were washed once in 50 mM Bis–Tris propane, pH 6.8, 20 mM KCl, 10 mM $MgCl_2$ (Buffer A). Cytoplasmic membranes containing Complex I was prepared as in,[52] except that Buffer A was used in all steps. The protein concentration was measured by the BCA method (Pierce) using bovine serum albumin as standard.

3.5. Enzyme activity measurement

3.5.1. WrbA activity

WrbA activity was measured in 20 mM potassium phosphate buffer, pH 7.2, at room temperature. The activity was monitored as NADH oxidation at 340 nm using $\varepsilon_{NADH} = 6.22\ mM^{-1}\ cm^{-1}$. The assay was performed in the presence of 100 μM FMN, using 200 μM NADH and 200 μM of the respective quinones.

3.5.2. Complex II activity

The SQR activity was determined spectroscopically by monitoring the reduction of DCPIP (2,6-dichlorophenolindophenol) as the decrease in the absorbance at 600 nm using $\varepsilon_{DCPIP} = 20.7\ mM^{-1}\ cm^{-1}$ as described in[39] using either phenazine methosulfate (PMS) or the respective quinones as primary electron acceptor. The following final concentration was used in the assays: 20 mg/mL DCPIP, 20 mM succinate, 3–5 mg SQR and 0,5 mg/mL PMS or 50 mM quinone. The reactions were done at room temperature in 50 mM potassium phosphate buffer pH 7,4 containing 1 mM potassium cyanide and 0.1 mM EDTA. The reaction was started by adding PMS or quinone. When indicated, the inhibitor 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) was added to 10 μM final concentration.

3.5.3. Complex I enzyme activity

The NADH:quinone reductase activity was measured as NADH oxidation at 340 nm ($\varepsilon_{NADH} = 6.22\ mM^{-1}\ cm^{-1}$) and 25 °C in 50 mM Tris–Cl, pH 8, 10 mM KCl, 5 mM $MgCl_2$, 0.2 μg/mL gramicidin A, 150 μM NADH and 100 μM electron acceptor. Apparent kinetic constants for the quinones were determined by varying the quinone concentration (0.2–3 K_m) while keeping the concentration of NADH constant (150 μM). Microcal Origin (Microcal Software, MA, USA) was used to plot the initial rates and fitting the Michaelis–Menten equation to the data for calculation of apparent K_m and V_{max}. When measuring inhibition with rotenone the bacterial membrane vesicles (100 μg protein/mL) were preincubated with 6 μM rotenone at 25 °C for 5 min prior to assay. Piericidin A (700 nM) was added to the membrane vesicles (4 mg protein / mL) and the sample was then incubated at 25 °C for 4 min before the quinone reductase assay. Protein concentration during the activity measurement was 0.05 mg/mL. Gramicidin (0.2 μg/mL) was always added to uncouple any transmembrane proton gradient that may arise during measurements. To rule out that the products formed after illumination of the azido quinones, *R. capsulatus* membranes were incubated with 50 μM previously illuminated AzQs and the NADH:O_2 reductase activity was subsequently measured as before. Activity measurements without additions and with the corresponding unmodified quinone were performed in parallel.

3.6. Photolabeling reactions

The WrbA sample (10 μM WrbA and when applicable 50 μM of the respective azidoquinones in a total volume of 500 μL 20 mM potassium phosphate buffer, pH 7.2) was placed on a concave glass plate mounted in an ice bath, at a distance of 7 cm from the mid-wavelength UV light source (UVM-57 (UVP Inc.)). A 100 μL sample

was extracted after each different time point of illumination. The samples were kept on ice in the dark until the entire series was collected. The enzyme activity was measured as described in Section 3.5.1, within 1 h after illumination. Each reaction contained 10 μL of illuminated enzyme.

The Complex II sample (5 μM purified Complex II in detergent, and when applicable from 10 to 75 μM of the different azidoquinones, in a total volume of 500 μL 50 mM potassium phosphate buffer with 1 mM EDTA, pH 7.4) was placed on a concave glass plate mounted in an ice bath, at a distance of 7 cm from the mid-wavelength UV light source. A 100 μL sample was extracted at each illumination time point in the series. The samples were kept on ice in the dark until the entire series was collected. The enzyme activity was measured within 1 h after illumination as described in Section 3.5.2, using Q_1 as primary electron acceptor.

Labeling of Complex I containing *R. capsulatus* membranes was performed in a quartz cuvette with a 5 mm light path. Cytoplasmic membranes were diluted in Buffer A to 1 mg/mL protein and pre-incubated with about 20 μM of the photoactive substrate for 5 min in the dark before illumination. The samples were illuminated with mid-wavelength UV light for up to 10 min at a 7 cm distance from the light source (UVM-57). The samples were kept at 4 °C throughout the procedure. NADH:quinone reductase activity was measured immediately after illumination at 340 nm (ε_{NADH} = 6.22 mM^{-1} cm^{-1}) and 25 °C in 50 mM Tris–Cl, pH 8.0, 10 mM KCl, 5 mM $MgCl_2$, 0.2 μg/mL gramicidin, 5 mM KCN, in the presence of 100 μM Q_1 and 150 μM NADH.

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Supplementary data

Supplementary data (HRMS of azido-Q_0 (compound **3**) post-illumination, with a tentative assignment of the main products formed. ^1H, ^{13}C, NMR spectra, and HRMS of the novel compounds **6** and **8**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.03.075.

References and notes

1. Darrouzet, E.; Moser, C. C.; Dutton, P. L.; Daldal, F. *Trends Biochem. Sci.* **2001**, *26*, 445.
2. Osyczka, A.; Moser, C. C.; Daldal, F.; Dutton, P. L. *Nature* **2004**, *427*, 607.
3. Tocilescu, M. A.; Fendel, U.; Zwicker, K.; Kerscher, S.; Brandt, U. *J. Biol. Chem.* **2007**, *282*, 29514.
4. Hägerhäll, C. *Biochim. Biophys. Acta* **1997**, *1320*, 107.
5. Abramson, J.; Riistama, S.; Larsson, G.; Jasaitis, A.; Svensson-Ek, M.; Laakkonen, L.; Puustinen, A.; Iwata, S.; Wikström, M. *Nat. Struct. Biol.* **2000**, *7*, 910.
6. Matsumoto, Y.; Murai, M.; Fujita, D.; Sakamoto, K.; Miyoshi, H.; Yoshida, M.; Mogi, T. *J. Biol. Chem.* **2006**, *281*, 1905.
7. Swem, L. R.; Gong, X.; Yu, C. A.; Bauer, C. E. *J. Biol. Chem.* **2006**, *281*, 6768.
8. Beyer, R. E.; SeguraAguilar, J.; DiBernardo, S.; Cavazzoni, M.; Fato, R.; Fiorentini, D.; Galli, M. C.; Setti, M.; Landi, L.; Lenaz, G. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 2528.
9. Yeh, J. I.; Chinte, U.; Du, S. C. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 3280.
10. Lummen, P. *Biochim. Biophys. Acta* **1998**, *1364*, 287.
11. Hollingworth, R. M.; Ahammadsahib, K. I.; Gadelhak, G.; McLaughlin, J. L. *Biochem. Soc. Trans.* **1994**, *22*, 230.
12. Rutherford, A. W.; Krieger-Liszkay, A. *Trends. Biochem. Sci.* **2001**, *26*, 648.
13. Sherer, T. B.; Richardson, J. R.; Testa, C. M.; Seo, B. B.; Panov, A. V.; Yagi, T.; Matsuno-Yagi, A.; Miller, G. W.; Greenamyre, J. T. *J. Neurochem.* **2007**, *100*, 1469.
14. Jung, K.; Reszka, R. *Adv. Drug Delivery Rev.* **2001**, *49*, 87.
15. Wong, T. W. L.; Yu, H. Y.; Kong, S. K.; Fung, K. P.; Kwok, T. T. *Life Sci.* **2000**, *67*, l1111.
16. Ohkura, K.; Lee, J. D.; Shimizu, H.; Nakano, A.; Uzui, H.; Horikoshi, M.; Fujibayashi, Y.; Yonekura, Y.; Ueda, T. *Mol. Cell. Biochem.* **2003**, *248*, 203.
17. Li, R. B.; Bianchet, M. A.; Talalay, P.; Amzel, L. M. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 8846.
18. Yu, C. A.; Gu, L. Q.; Yu, L. *Biochem. Biophys. Res. Commun.* **1982**, *105*, 624.
19. Lee, G. Y.; He, D. Y.; Yu, L.; Yu, C. A. *J. Biol. Chem.* **1995**, *270*, 6193.
20. Shenoy, S. K.; Yu, L.; Yu, C. A. *J. Biol. Chem.* **1997**, *272*, 17867.
21. Yang, X. D.; Yu, L.; He, D. Y.; Yu, C. A. *J. Biol. Chem.* **1998**, *273*, 31916.
22. Yu, L.; Yang, F. D.; Yu, C. A. *J. Biol. Chem.* **1985**, *260*, 963.
23. Heinrich, H.; Werner, S. *Biochemistry* **1992**, *31*, 11413.
24. Gong, X.; Xie, T.; Yu, L.; Hesterberg, M.; Scheide, D.; Friedrich, T.; Yu, C. A. *J. Biol. Chem.* **2003**, *278*, 25731.
25. Murai, M.; Sekiguchi, K.; Nishioka, T.; Miyoshi, H. *Biochemistry* **2009**, *48*, 688.
26. Sekiguchi, K.; Murai, M.; Miyoshi, H. *Biochim. Biophys. Acta* **2009**, *1787*, 1106.
27. Gu, L. Q.; Yu, L.; Yu, C. A. *Biochem. Biophys. Res. Commun.* **1983**, *113*, 477.
28. Sakamoto, K.; Nomura, K.; Miyoshi, H. *J. Pestic. Sci.* **2002**, *27*, 147.
29. Malen, C.; Lacoste, J.; Rue, B. *Fr. Demande.* **1992**, 41.
30. Liu, B.; Gu, L.; Zhang, J. *Recl. Trav. Chim. Pays-Bas* **1991**, *110*, 104.
31. Finley, K. T.. In *The Chemistry of Quinoid Compounds*; Wiley, 1988; Vol. 2, pp 608–609.
32. Silbert, L. S.; Swern, D. *J. Am. Chem. Soc.* **1959**, *81*, 2364.
33. Varma, R. S.; Kumar, D. *Catal. Lett.* **1998**, *53*, 225.
34. Monsef-Mirzal, P.; McWhinne, W. R. *Inorg. Chem. Acta* **1981**, *52*, 211.
35. Leyva, E.; Munoz, D.; Platz, M. S. *J. Org. Chem.* **1989**, *54*, 5938.
36. Grandori, R.; Carey, J. *Trends Biochem. Sci.* **1994**, *19*, 72.
37. Carey, J.; Brynda, J.; Wolfova, J.; Grandori, R.; Gustavsson, T.; Ettrich, R.; Smatanova, I. K. *Protein Sci.* **2007**, *16*, 2301.
38. Patridge, E. V.; Ferry, J. G. *J. Bacteriol.* **2006**, *188*, 3498.
39. Hägerhäll, C.; Aasa, R.; von Wachenfeldt, C.; Hederstedt, L. *Biochemistry* **1992**, *31*, 7411.
40. Lemma, E.; Hägerhäll, C.; Geisler, V.; Brandt, U.; von Jagow, G.; Kröger, A. *Biochim. Biophys. Acta* **1991**, *1059*, 281.
41. Dupuis, A.; Chevallet, M.; Darrouzet, E.; Duborjal, H.; Lunardi, J.; Issartel, J. P. *Biochim. Biophys. Acta* **1998**, *1364*, 147.
42. Grivennikova, V. G.; Roth, R.; Zakharova, N. V.; Hägerhäll, C.; Vinogradov, A. D. *Biochim. Biophys. Acta* **2003**, *1607*, 79.
43. Magnitsky, S.; Toulokhonova, L.; Yano, T.; Sled, V. D.; Hägerhäll, C.; Grivennikova, V. G.; Burbaev, D. S.; Vinogradov, A. D.; Ohnishi, T. *J. Bioenerg. Biomembr.* **2002**, *34*, 193.
44. Esposti, M. D.; Ghelli, A. *Biochem. Soc. Trans.* **1999**, *27*, 606.
45. Leif, H.; Sled, V. D.; Ohnishi, T.; Weiss, H.; Friedrich, T. *Eur. J. Biochem.* **1995**, *230*, 538.
46. Grandori, R.; Khalifah, P.; Boice, J. A.; Fairman, R.; Giovanielli, K.; Carey, J. *J. Biol. Chem.* **1998**, *273*, 20960.
47. Sakamoto, K.; Miyoshi, H.; Ohshima, M.; Kuwabara, K.; Kano, K.; Akagi, T.; Mogi, T.; Iwamura, H. *Biochemistry* **1998**, *37*, 15106.
48. Friedrich, T.; Brors, B.; Hellwig, P.; Kintscher, L.; Rasmussen, T.; Scheide, D.; Schulte, U.; Mänele, W.; Weiss, H. *Biochim. Biophys. Acta* **2000**, *1459*, 305.
49. Rappsilber, J.; Ishihama, Y.; Mann, M. *Anal. Chem.* **2003**, *75*, 663.
50. Hägerhäll, C.; Friden, H.; Aasa, R.; Hederstedt, L. *Biochemistry* **1995**, *34*, 11080.
51. Weaver, P. F.; Wall, J. D.; Gest, H. *Arch. Microbiol.* **1975**, *105*, 207.
52. Herter, S. M.; Schiltz, E.; Drews, G. *Eur. J. Biochem.* **1997**, *246*, 800.