Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Lampen, J. O., & Tkacz, J. S. (1975) *Biochem. Biophys. Res. Commun. 65*, 248.
Long, G. L., Belagaje, R. M., & MacGillivray, R. T. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5653.
MacGillivray, R. T. A., & Davie, E. W. (1984) *Biochemistry 23*, 1626.
Malhotra, O. M. (1982) *Biochim. Biophys. Acta 702*, 178.
Mameesh, M. S., & Johnson, B. C. (1959) *Proc. Soc. Exp. Biol. Med. 101*, 467.
Metta, V. C., Nash, L., & Johnson, B. C. (1961) *J. Nutr. 74*, 473.
Munns, T. W., Sims, H. F., & Strauss, A. W. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 1930 (Abstract).
O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007.
Owens, M. R., Miller, L. L., & Cimino, C. D. (1981) *Biochim. Biophys. Acta 676*, 365.
Patterson, J. E., & Geller, D. M. (1977) *Biochem. Biophys. Res. Commun. 74*, 1220.
Powell, J. R., Bretthauer, R. K., & Castellino, F. J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6836.
Robbins, P. W., Hubbard, S. C., Turco, S. J., & Wirth, D. F. (1977) *Cell (Cambridge, Mass.) 12*, 893.
Roth, M. G., Fitzpatrick, J. P., & Compans, R. W. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6430.
Rothman, J. E. (1981) *Science (Washington, D.C.) 213*, 1213.
Russell, J. H., & Geller, D. M. (1975) *J. Biol. Chem. 250*, 3409.
Shah, D. V., & Suttie, J. W. (1972) *Arch. Biochem. Biophys. 150*, 91.
Shah, D. V., Swanson, J. C., & Suttie, J. W. (1984) *Thromb. Res. 35*, 451.
Spering, R., Furie, B. C., Blumenstein, M., Keyt, B., & Furie, B. (1978) *J. Biol. Chem. 253*, 3898.
Steiner, D. (1976) in *Peptide Hormones* (Parsons, J., Ed.) pp 49–61, University Park Press, Baltimore, MD.
Stenflo, J. (1972) *J. Biol. Chem. 247*, 8167.
Struck, D. K., Siuta, P. B., Lane, M. D., & Lennarz, W. J. (1978) *J. Biol. Chem. 253*, 5332.
Suttie, J. W. (1973) *Science (Washington, D.C.) 179*, 192.
Suttie, J. W. (1980) *CRC Crit. Rev. Biochem. 8*, 191.
Suttie, J. W. (1983) in *Plasma Protein Secretion by the Liver* (Glaumann, H., Peters, T., Jr., & Redman, C., Eds.) pp 375–403, Academic Press, London.
Swanson, J. C., & Suttie, J. W. (1982) *Biochemistry 21*, 6011.
Takahashi, N. (1977) *Biochem. Biophys. Res. Commun. 76*, 1194.
Tarentino, A. L., & Plummer, T. H. (1982) *J. Biol. Chem. 257*, 10776.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350.
Warren, L. (1959) *J. Biol. Chem. 234*, 1971.
Willingham, A. K., Martin, S. L., Graves, C. B., Grabau, G. G., & Munns, T. W. (1980) in *Vitamin K Metabolism and Vitamin K-dependent Proteins* (Suttie, J. W., Ed.) pp 553–559, University Park Press, Baltimore, MD.

Effect of Alkyl Side Chain Variation on the Electron-Transfer Activity of Ubiquinone Derivatives†

Chang-An Yu,* Lianquan Gu, Yizhen Lin, and Linda Yu
Department of Biochemistry, Oklahoma State University, Stillwater, Oklahoma 74078
Received October 29, 1984

ABSTRACT: The effect of the alkyl side chain of the ubiquinone molecule on the electron-transfer activity of ubiquinone in mitochondrial succinate-cytochrome *c* reductase is studied by using synthetic ubiquinone derivatives that possess the basic ubiquinone structure of 2,3-dimethoxy-5-methyl-1,4-benzoquinone with different alkyl side chains at the 6-position. The alkyl side chains vary in chain length, degree of saturation, and location of double bonds. When a ubiquinone derivative is used as an electron acceptor for succinate-ubiquinone reductase, an alkyl side chain of six carbons is needed to obtain the maximum activity. However, when it serves as an electron donor for ubiquinol-cytochrome *c* reductase or as a mediator in succinate-cytochrome *c* reductase, an alkyl side chain of 10 carbons gives maximal efficiency. Introduction of one or two isolated double bonds into the alkyl side chain of the ubiquinone molecule has little effect on electron-transfer activity. However, a conjugated double bond system in the alkyl side chain drastically reduces electron-transfer efficiency. The effect of the conjugated double bond system on the electron-transferring efficiency of ubiquinone depends on its location in the alkyl side chain. When location is far from the benzoquinone ring, the effect is minimal. These observations together with the results obtained from photoaffinity-labeling studies lead us to conclude that flexibility in the portion of the alkyl side chain immediately adjacent to the benzoquinone ring is required for the electron-transfer activity of ubiquinone.

The essential role of ubiquinone (Q)[1] in mitochondrial and photosynthetic electron-transfer and energy conservation reactions has been well established (Ernster, 1976; Crane, 1977; Wraight, 1979; Trumpower, 1981). It is involved in both the redox reaction and proton translocation (Garland, 1976;

† This work was supported in part by the NIH (GM 30721) and Oklahoma Agricultural Experiment Station. The XL-300 NMR spectrometer was partially funded by the NSF.

[1] Abbreviations: PL, phospholipids; Q, ubiquinone; Q_0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; $Q_0(CH_2)_{10}OH$, 2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone; EDTA, ethylenediaminetetraacetic acid; DCCD, dicyclohexylcarbodiimide.

0006-2960/85/0424-3897$01.50/0

Mitchell, 1976; DiVirgillio & Azzone, 1982).

The naturally occurring ubiquinone in mitochondria or bacteria contains a prenyl side chain of 30 (Q_6) to 50 carbons (Q_{10}). Although the synthetic shorter side-chain Q homologues are active in electron-transfer reactions, the electron-transfer efficiency of these Q homologues varies significantly between different segments of the electron-transfer chain. Q_2 or its hydrogenated derivatives are fully active in the succinate oxidase system, whereas Q_4 or higher side-chain Q homologues are needed in the NADH oxidase system (Lenaz et al., 1968, 1978; Crane, 1977). Very little information regarding the molecular configuration of the prenyl side chain of the Q molecule is available. It was generally thought that the prenyl side chain exists in an extended straight-chain configuration (Crane, 1977; Boicelli et al., 1981), because the all-trans configuration of the prenyl side chain is thermodynamically favored. The results of differential scanning calorimetry studies of phospholipid vesicles formed with a high molar ratio of Q to phospholipids also favor a prenyl side chain in the extended straight-chain configuration (Katsikas & Quinn, 1981, 1983a,b). Recent results obtained from the study of the Q–protein interactions using synthetic arylazido-Q (Yu & Yu, 1982) and azido-Q derivatives (Yu et al., 1985) have raised a question concerning this extended prenyl side chain configuration. Two protein subunits (M_r 17 000 and 37 000) of ubiquinol-cytochrome *c* reductase were heavily labeled when either the azido-Q or arylazido-Q derivative was used as a photoaffinity-labeling reagent. Although the location of the photoactivated azido group in the arylazido-Q derivative is more than 15 carbons away from the benzoquinone ring, it labeled the same protein subunits as did the azido-Q derivative in which the azido group is located on the benzoquinone ring. One plausible explanation is that the prenyl side chain of Q in the protein-bound form is in close proximity to the benzoquinone ring. To test this possibility, we have synthesized various Q derivatives having different degrees of rigidity in the alkyl side chains. The comparative effectiveness of these Q derivatives in restoring activity to the Q-depleted succinate-cytochrome *c* reductase has been investigated. If flexibility in the alkyl side chain of the Q molecule is needed for the Q–protein interaction, an essential step for the Q-mediated electron-transfer reaction, then any Q derivative with a rigid alkyl side chain would have less electron-transfer activity than Q derivatives with flexible alkyl side chains. When the location of the rigid portion in the alkyl side chain is varied, the part of the alkyl side chain in which flexibility is essential can be revealed. Herein we report the synthesis and spectral properties of Q derivatives with various alkyl side chains and the effect of these variations on the electron-transfer activity of ubiquinone.

Experimental Procedures

Materials. $Pd/CaCO_3$, Pd/C, and other organic chemicals used in synthesis were from Aldrich or Eastman, the highest purity available; cytochrome *c* and Florisil were from Sigma; asolectin was from Associated Concentrates; silica gel 1-H plates with SiO_2 as binder were from Supelco.

Enzyme Preparation. Succinate-Q reductase (Yu & Yu, 1982a), ubiquinol-cytochrome *c* reductase (Yu & Yu, 1980), succinate-cytochrome *c* reductase (Yu & Yu, 1982a), and its Q- and PL-depleted forms (Yu et al., 1978) were prepared as reported previously.

The electron-acceptor activity of ubiquinone derivatives was measured by using succinate-Q reductase. The reaction mixture, in a final volume of 1 mL, contained 50 μmol of sodium/potassium phosphate buffer, pH 7.0, 20 μmol of succinate, 10 nmol of EDTA, 0.1 mg of Triton X-100, 52 nmol of 2,6-dichlorophenolindophenol (DCIP), and 30 nmol of Q derivatives. The reduction of DCIP was followed spectrophotometrically by measuring the decrease in the absorption at 600 nm after addition of succinate-Q reductase (1–2 μg). A millimolar extinction coefficient of 21 was used for DCIP in the activity calculation.

The electron-donating activity of Q derivatives was assayed with ubiquinol-cytochrome *c* reductase. The assay mixture, in a final volume of 1 mL, contained 100 μmol of sodium/potassium phosphate buffer, pH 7.0, 300 nmol of EDTA, 100 nmol of cytochrome *c*, and 30 nmol of reduced Q derivatives. The ubiquinol-cytochrome *c* reductase activity was measured by following the reduction of cytochrome *c* at 550 nm, after addition of enzyme (0.1–0.5 μg). The nonenzymatic reduction of cytochrome *c* by reduced Q derivatives was recorded before the addition of enzyme and was subtracted from the value obtained after addition of enzyme. A millimolar extinction coefficient of 18.5 for the difference between reduced and oxidized cytochrome *c* at 550 nm was used for the activity calculation.

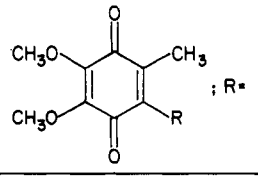
For determination of the electron-mediator activity of Q derivatives, 0.1 mL of the Q- and PL-depleted succinate-cytochrome *c* reductase, 6 mg/mL (24 μM cytochrome *b*), in 50 mM sodium/potassium phosphate buffer, pH 7.4, containing 10% glycerol was mixed with 30 nmol of Q derivatives in 2 μL of ethanol. After incubation for 2 min at 0 °C, 30 μL of the asolectin micellar solution (10 mg/mL in H_2O) was added, and the mixture was then diluted with 0.2 mL of 50 mM phosphate buffer, pH 7.4. The succinate-cytochrome *c* reductase activity was assayed after the mixture was incubated at 0 °C for 2 h. The assay conditions are similar to that of ubiquinol-cytochrome *c* reductase except that succinate (20 mM) was used as substrate, and the pH of the assay mixture was at 7.4.

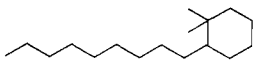
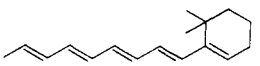
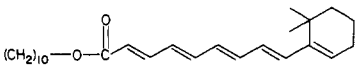
Protein was estimated by the biuret method in the presence of hydrogen peroxide (Yanetani, 1961) with crystalline bovine serum albumin as standard.

Spectral Measurement. The absorption spectra and spectrophotometric assays of enzymatic activities were done on a Cary spectrophotometer, Model 219, at room temperature. NMR spectra were measured on either a Hitachi Perkin-Elmer Model R24B or a 100-MHz Varian XL-100 NMR spectrometer. Mass spectra were measured with a high-resolution CEC21-110 B mass spectrometer with Nora data acquisition system. IR spectra were done on a Perkin-Elmer grating infrared spectrophotometer, Model 457.

Organic Syntheses. 2,3-Dimethoxy-5-methyl-1,4-benzoquinone, 2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone, 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone, and 2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone [$Q_0(CH_2)_{10}OH$] were synthesized according to the methods described previously (Yu & Yu, 1982b). Synthesis of 2,3-dimethoxy-5-methyl-6-[10-(retinoyloxy)-decyl]-1,4-benzoquinone was carried out by the esterification of 2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone with retinoic acid. The esterification reaction was catalyzed by DCCD (Yu & Yu, 1982b). Syntheses of 2,3-dimethoxy-5-methyl-6-retinyl-1,4-benzoquinone, 2,3-dimethoxy-5-methyl-6-(hexa-2,4-dienyl)-1,4-benzoquinone, 2,3-dimethoxy-5-methyl-6-(hepta-2,4-dienyl)-1,4-benzoquinone, 2,3-dimethoxy-5-methyl-6-(nona-2,4-dienyl)-1,4-benzoquinone, and 2,3-dimethoxy-5-methyl-6-(3,7-dimethylocta-2,4,6-trienyl)-1,4-benzoquinone were carried out by alkylation of 2,3-

Table I: Chemical Structure, Spectral Properties, and Electron-Transfer Activities of Ubiquinone Derivatives



compounds		spectral data		electron transfer activities[a]	
		H^1 NMR ($CDCl_3$)	$UV_{max}^{95\%EtOH}$ (nm)	as acceptor [μmol of Succ/(mg·min)]	as donor [μmol of c/(mg·min)]
I	CH_3	4.00 (s, 6), 2.02 (s, 6)	ox. 276, red. 289	0.2	1.0
II	CH_2CH_3	4.00 (s, 6), 2.50 (t, 2), 2.03 (s, 3), 1.05 (t, 3)	ox. 276, red. 289	1.1	3.0
III	$CH_2CH_2CH_3$	4.00 (s, 6), 2.46 (t, 2), 2.02 (s, 3), 1.45 (m, 2), 0.97 (t, 3)	ox. 277, red. 289	2.0	4.0
IV	$CH_2CH_2CH_2CH_3$	4.00 (s, 6), 2.47 (t, 2), 2.02 (s, 3), 1.39 (m, 4), 0.94 (t, 3)	ox. 278, red. 289	5.1	18.0
V	$CH_2CH_2CH_2CH_2CH_2CH_3$	4.00 (s, 6), 2.40 (t, 2), 2.01 (s, 3), 1.27 (br, s, 8), 0.88 (t, 3)	ox. 279, red. 288	16.8	64.0
VI	$CH_2CH{=}CHCH{=}CHCH_3$	6.02 (m, 2), 5.40–5.70 (br, m, 2), 4.00 (s, 6), 3.22 (d, 2), 2.01 (s, 3), 1.71 (d, 3)	ox. 275, red. 289	10.2	20.0
VII	$CH_2CH_2CH_2CH_2CH_2CH_2CH_3$	4.00 (s, 6), 2.40 (t, 2), 2.01 (s, 3), 1.27 (br, s, 10), 0.88 (t, 3)	ox. 279, red. 289	17.0	99.2
VIII	$CH_2CH{=}CHCH{=}CHCH_2CH_3$	6.07 (br, m, 2), 5.40–5.80 (br, m, 2), 4.00 (s, 6), 3.22 (d, 2), 2.01 (m, 5), 0.98 (t, 3)	ox. 275, red. 289	10.6	48.1
IX	$CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_3$	4.00 (s, 6), 2.40 (t, 2), 2.01 (s, 3), 1.27 (br, s, 14), 0.99 (t, 3)	ox. 279, red. 280	17.0	154.0
X	$CH_2CH{=}CHCH{=}CHCH_2CH_2CH_2CH_3$	6.07 (br, m, 2), 5.40–5.80 (br, m, 2), 4.00 (s, 6), 3.22 (d, 2), 2.01 (m, 5), 1.40 (br, m, 4), 0.90 (t, 3)	ox. 275, red. 289	10.6	136.0
XI	$CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_3$	4.00 (s, 6), 2.40 (t, 2), 2.01 (s, 3), 1.27 (br, 16), 0.88 (t, 3)	ox. 279, red. 289	17.0	160.0
XII	$CH_2CH{=}C(CH_3)CH_2CH_2CH{=}C(CH_3)_2$	5.00 (t, 2), 4.00 (s, 6), 3.20 (d, 2), 2.03 (m, 7), 1.65 (t, 9)	ox. 276, red. 289	17.0	160.0
XIII	$CH_2CH_2CH(CH_3)CH_2CH_2CH_2CH(CH_3)_2$	4.00 (s, 6), 2.40 (t, 2), 2.01 (s, 3), 1.22 (br, m, 10), 0.90 (t, 9)	ox. 279, red. 289	17.0	159.9
XIV	$CH_2CH{=}C(CH_3)CH{=}CHCH{=}C(CH_3)_2$	5.86–6.40 (br, m, 3), 5.24 (t, 1), 4.00 (s, 6), 3.35 (d, 2), 2.03 (s, 3), 1.84 (m, 9)	ox. 278 and 286, red. 278 and 290	11.0	72.1
XV		4.00 (s, 6), 2.44 (t, 2), 2.02 (s, 3), 0.88–1.76 (br, m, 33)	ox. 279, red. 289	6.0	120.2
XVI		6.14–6.80 (m, 5), 5.36 (m, 1), 4.00 (s, 6), 3.36 (t, 2), 2.10 (s, 3), 2.02 (d, 6), 1.12–1.72 (br, m, 9), 1.04 (s, 6)	ox. 331 and 285	3.0	14.0
XVII		7.02 (m, 1), 6.16–6.32 (br, m, 4), 5.82 (s, 1), 4.00 (s, 6), 3.50 (t, 2), 2.45 (t, 2), 2.38 (s, 3), 2.02 (d, 9), 1.30–1.72 (br, m, 25), 1.04 (s, 6)	ox. 350 and 280	8.9	126.2

[a] Both electron-donor and -acceptor activities given are the maximal activity.

dimethoxy-5-methyl-1,4-benzoquinol with corresponding alcohols. The alkylation was promoted by $KHSO_4$ and elevated temperature (72 °C). Hepta-2,4-dienal and nona-2,4-dienal are commerically available and were converted to hepta-2,4-dien-1-ol and nona-2,4-dien-1-ol by treatment with $NaBH_4$ in ethanolic solution. 3,7-Dimethylocta-2,4,6-trien-1-ol was prepared from 3,7-dimethylocta-2,4,6-trienal by reduction with $NaBH_4$ in ethanolic solution. 3,7-Dimethylocta-2,4,6-trienal was prepared from 2,6-dimethylhepta-2,5-dien-4-one via 2,6-dimethylhepta-2,5-dien-4-ol and 2,6-dimethylhepta-1,3,5-triene by the process of reduction, dehydration, and condensation. 2,3-Dimethoxy-5-methyl-6-hexyl-1,4-benzoquinone, 2,3-dimethoxy-5-methyl-6-heptyl-1,4-benzoquinone, 2,3-dimethoxy-5-methyl-6-nonyl-1,4-benzoquinone, and 2,3-dimethoxy-5-methyl-6-(3,7-dimethylocytl)-1,4-benzoquinone were prepared from 2,3-dimethoxy-5-methyl-6-(hexa-2,4-dienyl)-1,4-benzoquinone, 2,3-dimethoxy-5-methyl-6-(hepta-2,4-dienyl)-1,4-benzoquinone, 2,3-dimethoxy-5-methyl-6-(nona-2,4-dienyl)-1,4-benzoquinone, and 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone, respectively, by catalytic hydrogenation. Syntheses of 2,3-dimethoxy-5-methyl-6-acetyl-1,4-benzoquinone, 2,3-dimethoxy-5-methyl-6-propyl-1,4-benzoquinone, and 2,3-dimethoxy-5-methyl-6-butyl-1,4-benzoquinone were carried out essentially according to the method reported by Wan et al. (1975) using diacetyl, dipropanoyl, dibutanoyl, and dipentanoyl peroxide, respectively. The peroxides were prepared from their corresponding acids by the method reported by Silbert & Swern (1959). 2,3-Dimethoxy-5,6-dimethyl-1,4-benzoquinone was synthesized according to the method of Fieser & Chang (1942) using lead tetraacetate.

The chemical structures of the synthesized Q derivatives were identified by the spectral properties of NMR, IR, mass, and UV absorption.

Results and Discussion

Synthesis, Structure, and Spectral Properties of Q Derivatives. Table I summarizes the structural and spectral

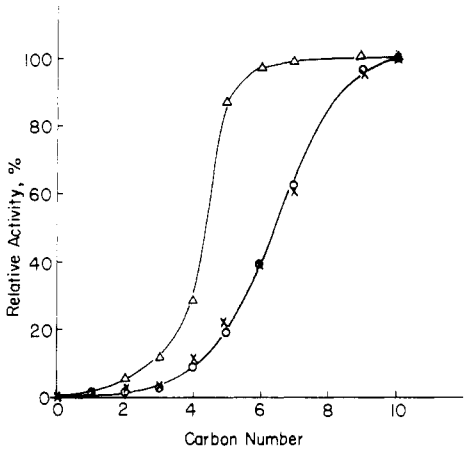


FIGURE 1: Alkyl chain length dependent electron-transfer activity of synthetic ubiquinone derivatives. (Δ) As an electron acceptor for succinate-Q reductase; (O) as an electron mediator in succinate-cytochrome *c* reductase; (×) as an electron donor for ubiquinol-cytochrome *c* reductase. The carbon number refers to the normal alkyl groups except for C_5 in which 3-methyl-2-butenyl is used. One hundred percent activity for succinate-Q, ubiquinol-cytochrome *c*, and succinate-cytochrome *c* reductases are 17 μmol of succinate oxidized, 80 μmol of ubiquinol oxidized, and 3.6 μmol of succinate oxidized per minute per milligram of protein at 23 °C, respectively.

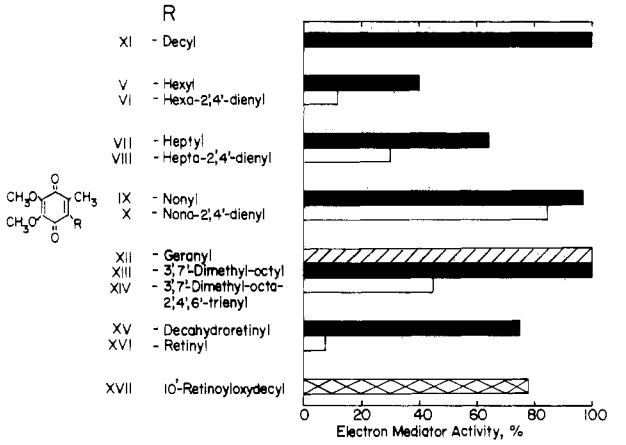


FIGURE 2: Comparison of the electron-mediator activity of ubiquinone derivatives with saturated and unsaturated alkyl side chains. The relative activity refers to that restored from the Q- and phospholipid-depleted succinate-cytochrome *c* reductase upon addition of Q derivatives and phospholipids. One hundred percent activity is equal to 3.6 μmol of succinate oxidized per minute per milligram of protein at 23 °C.

properties of Q derivatives synthesized for this study. These derivatives all contain a common structure of 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q_0). Thus, structural variations in these Q derivatives are limited to the substituent at the 6-position of the 1,4-benzoquinone ring. These Q derivatives can be divided into three groups according to the structure of their alkyl side chain: Q_0 with saturated alkyl side chains, Q_0 with unsaturated alkyl side chains, and the esters of $Q_0(CH_2)_{10}OH$. The key reactions involved in the synthesis of these Q derivatives are the acid-catalyzed coupling of Q_0 with alcohols, the radical coupling of Q_0 with dialkyl peroxides, and the esterification of $Q_0C_{10}OH$ with acids. The details of these reactions were reported previously (Yu & Yu, 1982b) and were adapted, without modifications, for the syntheses of Q derivatives used in this study. The methods used for purification of the synthetic compounds are solvent extraction, column chromatography, and thin-layer chromatography. The purity and chemical structure determinations are based on spectral data of NMR, IR, mass, and UV absorption. Although yields, in some cases, were relatively low, no effort was made to improve them because our main concern is with the biological properties of these compounds and the amount needed for biological studies is rather small.

Effect of the Length of the Alkyl Side Chain on Electron-Transfer Activity of Q Derivatives. Three kinds of activities can be used to measure the effectiveness of ubiquinone derivatives in electron-transfer reactions. These are the ability (1) of Q derivatives to serve as an electron acceptor, as in succinate-Q or NADH-Q reductase, (2) of reduced Q derivatives to serve as an electron donor, as in ubiquinol-cytochrome *c* reductase, and (3) of Q derivatives to serve as electron mediators between two electron-transfer complexes, as in succinate-cytochrome *c* or NADH-cytochrome *c* reductase.

Several reports (Land et al., 1974; Crane, 1977; Boicelli et al., 1981) have dealt with the effectiveness of Q homologues (the Q molecule with various units of isoprenoid side chains) in restoring succinate oxidase and NADH oxidase activities of the Q-depleted mitochondria. A systematic study of Q derivatives that possess alkyl side chains of 1–10 carbons, serving as electron acceptors, donors, and mediators for individual complexes, has not been made. These short side chain Q derivatives are very important in studying the Q–protein interaction because, as described in the following section, the electron-transfer activity of Q derivatives depends on the flexibility of the portion of the alkyl group immediately adjacent to the benzoquinone ring. This portion involves less than 10 carbons.

Figure 1 shows the electron-transfer activities of Q derivatives with alkyl side chains of 1–10 carbons. When Q derivatives are used as electron acceptors for succinate-Q reductase, six carbons in the alkyl side chain is enough to obtain maximal activity. However, when these Q derivatives are measured for their ability to restore the electron-transfer activity to the Q-depleted succinate-cytochrome *c* reductase or to donate the electron to ubiquinol-cytochrome *c* reductase, at least a 10-carbon alkyl side chain is needed for maximal activity. The Q derivatives with an alkyl side chain of 10–50 carbons should be fully active. Under the reported experimental conditions, maximal activity was observed for alkyl side chains between 10 and 30 carbons; beyond 30 carbons activity decreased (Yu et al., 1978). This is obviously not due to the ineffectiveness of higher side-chain Q homologues such as Q_{10} but rather to the low solubility of Q_{10} in aqueous solution.

The Q derivatives with saturated alkyl side chains of the same number of carbons show a similar ability to restore activity to the Q-depleted succinate-cytochrome *c* reductase, regardless of whether they are in a straight-chain or in a branched-chain configuration. For example, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (XI) has the same electron-transfer efficiency as does 2,3-dimethoxy-5-methyl-6-(3,7-dimethyloctyl)-1,4-benzoquinone (XIII) (see Figure 2).

Effect of Flexibility of the Alkyl Side Chain on the Electron-Transfer Activity of Q Derivatives. Figure 2 compares the electron-transfer activity of Q derivatives with different flexibilities of their alkyl side chains. The activity of Q-depleted succinate-cytochrome *c* reductase restored by addition of these Q derivatives is compared to that restored by Q_2. Under the experimental conditions, more than 85% of the original succinate-cytochrome *c* reductase activity [4 μmol of succinate oxidized min^{-1} (mg of protein)$^{-1}$ at 23 °C] is restored by the addition of Q_2.

Introduction of isolated double bonds to the alkyl side chain at C_2' has no effect on the electron-transfer activity compared to the saturated counterpart. For example, 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone (XII) has the same

activity as its hydrogenated alkyl side chain derivative, 2,3-dimethoxy-5-methyl-6-(3,7-dimethyloctyl)-1,4-benzoquinone (XIII). The lack of change in electron-transfer activity upon introduction of isolated double bonds to the alkyl side chain is explained by the fact that this modification causes little change in the flexibility of the alkyl side chain. On the other hand, a double bond in the alkyl side chain at C_1' results in drastic reduction of the electron-transfer activity of Q derivatives. Morimoto & Imada (1972) reported that when the first double bond in the prenyl side chain of Q_7 was moved from C_2' to C_1', the effectiveness of this Q derivative in restoring succinate oxidase activity of a Q-depleted mitochondrial preparation was decreased by 93%, compared to that of Q_7. This observation is explained by the formation of a conjugated system between the alkyl side chain and the benzoquinone ring, thus decreasing the flexibility of the alkyl side chain near the benzoquinone ring.

In contrast to little effect of isolated double bonds in C_2', introduction of a conjugated double-bond system at C_2' drastically reduces the electron-transfer activity of Q derivatives. The degree of reduction of activity depends not only on the number but also on the location of conjugated double bonds in the alkyl side chain. Figure 2 shows that introduction of a pair of conjugated double bonds in the alkyl side chain to form the 2,4-diene greatly reduces the electron-mediator activity relative to the corresponding saturated derivatives. This reduction of activity varies inversely with the carbon chain length of the 2,4-diene. When the hexyl side chain of Q derivative (V) is converted to a hexa-2,4-dienyl group (VI), a 70% decrease in activity is observed. Converting the heptyl side chain of Q derivative (VII) to the hepta-2,4-dienyl group (VIII) and the nonyl side chain (IX) to the nona-2,4-dienyl group (X) results in a 54% and 13% reduction of activity, respectively.

The effectiveness of Q derivatives as electron mediators in succinate-cytochrome *c* reductase decreases as the degree of conjugation in the alkyl side chain increases. Whereas the pair of conjugated double bonds in the nona-2,4-dienyl group (X) causes a 13% less in activity, when a third double bond is added to the geranyl group (XII) to form a conjugated triene system, 3,7-dimethylocta-2,4,6-trienyl group (XIV), a more than 50% reduction in activity is observed. Moreover, a five conjugated double-bond system in the alkyl side chain gives more than 90% reduction in activity. For instance, 2,3-dimethoxy-5-methyl-6-retinyl-1,4-benzoquinone (XVI) has 7% activity (compared to Q_2), whereas the fully hydrogenated retinyl side chain derivative (XV) has about 75% activity.

Although 2,3-dimethoxy-5-methyl-6-retinyl-1,4-benzoquinone (XVI) has less than 10% of the activity of Q_2, 2,3-dimethoxy-5-methyl-6-[10-(retinoyloxy)decyl]-1,4-benzoquinone (XVII), synthesized by esterification of the retinoic acid with 2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone, shows 82% activity compared to that of Q_2. This result demonstrates that the effect of the conjugated system on the electron-transfer activity varies greatly with its location in the alkyl side chain. When the conjugated system is located far from the benzoquinone ring, the effect is minimal.

These results together with results obtained from photoaffinity studies using arylazido-Q (Yu & Yu, 1982) and azido-Q (Yu et al., 1985) as the labeling reagents lead us to conclude that flexibility in the portion of the alkyl side chain near the benzoquinone ring is a requirement for the electron-transfer activity of Q derivatives. This conclusion is in agreement with our previous assessment (Yu & Yu, 1981) that the active species of Q molecules in electron-transfer reactions are those bound to specific proteins. Apparently a specific structure is required for Q molecules to react with the Q-binding site. Any Q-derivative which does not have the required structure will not have electron transfer activity.

The structural requirement for Q to serve as an electron acceptor is less specific than those needed for Q to function as an electron mediator. As described above, the carbon chain length required for full activity of succinate-Q reductase is much shorter than that required for succinate-cytochrome *c* reductase. Introduction of a pair or more of conjugated double bonds decreases the electron-transfer activity by about 30% compared to the saturated side chain. Even when the decahydroretinyl side chain (XV) is converted to the retinyl group (XVI), only a 40% reduction in activity results. However, these changes (XV → XVI) result in a more than 90% reduction in activity as an electron mediator in the succinate-cytochrome *c* reductase system. One explanation is that added Q derivatives accept electrons from endogenously bound Q, when they act as electron acceptors, without specific binding between these added Q derivatives and protein. Alternatively, the results suggest different Q-binding environments in succinate-Q and ubiquinol-cytochrome *c* reductases. Although the electron transfer between ubiquinol and ubiquinone can not take place when both are in the free form, it is possible that electron transfer occurs readily when one of them is in the protein-bound form, particularly for the one-electron-transfer step. The involvement of the ubisemiquinone radical in electron-transfer reactions has been well documented in mitochondrial and photosynthetic systems (Backstrom et al., 1970; Petty & Dutton, 1976; Konstantinov, 1977; Wraight 1979; Yu et al., 1980).

Since the protein-bound Q constitutes only a small portion of the total Q available in the mitochondrial inner membrane or bacterial plasma membrane, kinetic studies of the redox reaction of Q using the mitochondrial membranes revealed mainly the behavior of the free form of Q (Kroger & Klingenberg, 1977). Since thermal transition studies (Boicelli et al., 1981; Katsikas & Quinn, 1983b) of the phospholipid–Q vesicle formed in the absence of protein can only provide information for the molecular configuration of the free Q molecule, the existence of the extended straight alkyl side chain configuration in the Q molecule, supported by the thermotransition studies, is not in conflict with the idea that a flexible alkyl side chain, near the benzoquinone ring, is needed in the bound form of Q. The coexistence of the protein-bound and free forms of Q in mitochondria can explain the unusual behavior of the reduction or oxidation (Chance et al., 1969; Kroger & Klingenberg, 1973) kinetics of Q. The slower kinetics of Q reduction, compared to that of other redox components, is expected if one measures the major free form of Q in the mitochondrial membrane. Redox kinetics of Q comparable to those of other redox components would be obtained if only the protein-bound form of Q were measured.

Registry No. I, 483-54-5; II, 91971-27-6; III, 96706-27-3; IV, 96706-28-4; V, 95268-75-0; VI, 95268-74-9; VII, 93157-25-6; VIII, 95268-76-1; IX, 95169-01-0; X, 95268-77-2; XI, 55486-00-5; XII, 7704-04-3; XIII, 74391-72-3; XIV, 95268-78-3; XV, 96728-94-8; XVI, 96706-29-5; XVII, 96706-30-8; 2,3-dimethoxy-5-methyl-1,4-benzoquinol, 96706-31-9; hepta-2,4-dienal, 5910-85-0; nona-2,4-dienal, 6750-03-4; hepta-2,4-dien-1-ol, 62488-55-5; nona-2,4-dien-1-ol, 62488-56-6; 3,7-dimethylocta-2,4,6-trien-1-ol, 96706-32-0; 3,7-dimethylocta-2,4,6-trienol, 64937-68-4; 2,6-dimethylhepta-2,5-dien-4-ol, 58210-14-3; 2,6-dimethylhepta-1,3,5-triene, 928-67-6; 2,6-dimethylhepta-2,5-dien-4-one, 504-20-1; succinate-Q reductase, 9028-11-9; ubiquinol-cytochrome *c* reductase, 9027-03-6; succinate-cytochrome *c* reductase, 9028-10-8; 2,3-dimethoxy-5-methyl-

6-acetyl-1,4-benzoquinone, 96706-33-1.

References

Backstrom, D., Norling, B., Ehrenberg, A., & Ernster, L. (1970) *Biochim. Biophys. Acta 197*, 108–111.

Boicelli, C. A., Ramponi, C., Casali, E., & Masotti, L. (1981) *Membr. Biochem. 4*, 105–118.

Chance, B., Azzi, A., Lee, J. Y., Lee, C. P., & Mela, L. (1969) in *Mitochondria-Structure and Function* (Ernster, L., Ed.) pp 233–273, Academic Press, New York.

Crane, L. L. (1977) *Annu. Rev. Biochem. 46*, 439–469.

Di Virgilio, F., & Azzone, G. F. (1982) *J. Biol. Chem. 257*, 4106–4113.

Ernster, L. (1976) in *Biomedical and Clinical Aspects of Coenzyme Q* (Folkers, K., & Yamamura, Y., Eds.) pp 15–19, Elsevier, Amsterdam, Oxford, New York.

Fieser, L. F., & Chang, F. C. (1942) *J. Am. Chem. Soc. 64*, 2043–2052.

Garland, P. B. (1976) in *Biomedical and Clinical Aspects of Coenzyme Q* (Folkers, K., & Yamamura, Y., Eds.) pp 23–27, Elsevier, Amsterdam, Oxford, New York.

Katsikas, H., & Quinn, P. J. (1981) *FEBS Lett. 133*, 230–234.

Katsikas, H., & Quinn, P. J. (1983a) *J. Bioenerg. Biomembr. 15*, 67–79.

Katsikas, H., & Quinn, P. J. (1983b) *Eur. J. Biochem. 131*, 607–612.

Konstantinov, A. A., & Ruuge, E. K. (1977) *FEBS Lett. 81*, 137–141.

Kroger, A., & Klingenberg, M. (1973) *Eur. J. Biochem. 34*, 358–368.

Land, B., Burger, G., & Bandlow, W. (1974) *Biochim. Biophys. Acta 368*, 71–85.

Lenaz, G., Daves, D. G., & Folkers, K. (1968) *Arch. Biochem. Biophys. 169*, 539–550.

Lenaz, G., Landi, L., Cabrini, L., Sechi, A. M., & Ozawa, T. (1978) *Biochem. Biophys. Res. Commun. 85*, 1047–1055.

Mitchell, P. (1976) *J. Theor. Biol. 62* 327–367.

Morimoto, H., & Imada, I. (1972) *Biochim. Biophys. Acta 275*, 10–17.

Petty, K. M., & Dutton, L. (1976) *Arch. Biochem. Biophys. 172*, 335–345.

Silbert, L. S., & Swern, D. (1959) *J. Am. Chem. Soc. 81*, 2364–2367.

Trumpower, B. L. (1981) *J. Bioenerg. Biomembr. 13*, 1–23.

Wan, Y. P., Williams, R. H., Folkers, K., Leung, K. H., & Racker, E. (1975) *Biochem. Biophys. Res. Commun. 63*, 11–15.

Wraight, C. A. (1979) *Photochem. Photobiol. 30*, 767–776.

Yonetani, T. (1961) *J. Biol. Chem. 236*, 1680–1688.

Yu, C. A., & Yu, L. (1981) *Biochim. Biophys. Acta 639*, 99–128.

Yu, C. A., & Yu, L. (1982a) *J. Biol. Chem. 257*, 2016–2021.

Yu, C. A., & Yu, L. (1982b) *Biochemistry 21*, 4096–4101.

Yu, C. A., Nagaoka, S., Yu, L., & King, T. E. (1980) *Arch. Biochem. Biophys. 204*, 59–70.

Yu, L., & Yu, C. A. (1982) *J. Biol. Chem. 257*, 10215–10221.

Yu, L., Yu, C. A., & King, T. E. (1978) *J. Biol. Chem. 253*, 2657–2663.

Yu, L., Yang, E.-D., & Yu, C. A. (1985) *J. Biol. Chem. 260*, 936–973.

Structural and Functional Characterization of the Inhibition of Urokinase by α_2-Macroglobulin†

David L. Straight, M. Alycia Hassett, and Patrick A. McKee*

Howard Hughes Medical Institute Laboratories, Department of Medicine, and Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Received December 3, 1984

ABSTRACT: We have investigated the interaction of α_2-macroglobulin (α_2M) with the serine proteinase urokinase, an activator of plasminogen. Urokinase formed sodium dodecyl sulfate stable complexes with purified α_2M and with α_2M in plasma. These complexes could be visualized after polyacrylamide gel electrophoresis by protein blots using ^{125}I-labeled anti-urokinase antibody or by fibrin autography, a measure of fibrinolytic activity. According to gel electrophoretic analyses under reducing conditions, urokinase cleaved α_2M subunits and formed apparently covalent complexes with α_2M. Urokinase cleaved only about 60% of the α_2M subunits maximally at a mole ratio of 2:1 (urokinase:α_2M). Binding of urokinase to α_2M protected the urokinase active site from inhibition by antithrombin III–heparin and inhibited, to a significant extent, plasminogen activation by urokinase. Reaction of urokinase with α_2M caused an increase in intrinsic protein fluorescence and, thus, induced the conformational change in α_2M that is characteristic of its interactions with active proteinases. Our results indicate that both in plasma and in a purified system the α_2M–urokinase reaction is functionally significant.

α_2-Macroglobulin (α_2M)[1] has been shown to bind and inhibit a wide variety of proteinases, while leaving the active site of the proteinase free to attack small substrates (Mehl et al., 1964; Barrett & Starkey, 1973; Rinderknecht et al., 1975). The proteinase cleaves some or all of the four α_2M subunits (M_r ~185 000) during binding and is subsequently protected from other macromolecular inhibitors (Haverback et al., 1962; Ganrot, 1966). Binding of the proteinase to α_2M is considered to be essentially irreversible under nondenaturing conditions

† This work was supported in part by National Heart, Lung, and Blood Institute Grant HL-15615 (to P.A.K.) and a North Carolina Heart Association Grant-in-Aid (to M.A.H.).

* Address correspondence to this author at the Department of Medicine, Duke University Medical Center. He is an Investigator of the Howard Hughes Medical Institute.

[1] Abbreviations: α_2M, α_2-macroglobulin; SDS, sodium dodecyl sulfate; ATIII, antithrombin III; IgG, immunoglobulin G; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/85/0424-3902$01.50/0