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STUDY OF THE PROTEIN-UBIQUINONE INTERACTION IN SUCCINATE-CYTOCHROME c REDUCTASE WITH AZIDO-UBIQUINONE DERIVATIVES

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SUMMARY-- Several azido-ubiquinones have been synthesized for the study of protein-ubiquinone interaction in succinate-cytochrome c reductase. In the absence of light, azido-ubiquinones are partially effective in restoring enzymatic activity to ubiquinone- and phospholipid-depleted reductase and the binding of azido-ubiquinones can be partially reversed by 5-(10-bromodecyl)-ubiquinone. When 2-azido-3-methoxy-5-geranyl-6-methyl-1,4-benzoquinone reactivated reductase is illuminated with long wavelength UV light, a complete and irreversible innibition is observed. This specific photo-inactivation, exerted only by 2-azido-3-methoxy-5-geranyl-6-methyl-1,4-benzoquinone, and not by other azido-ubiquinone derivatives, is evidence for the existence of a specific benzoquinone ring binding site in the enzyme.

The participation of ubiquinone (Q) in the electron transfer reaction in succinate-cytochrome c reductase of the mitochondrial respiratory chain is well established (1, 2), as is the existence of specific Q-binding proteins in this segment (3-8). The active species of Q in electron transfer is a Q:protein complex. Evidence in support of a specific Q:protein interaction includes the spectral shift of Q upon interaction with protein (9), specific labeling of the protein subunit of succinate-Q reductase (9) and ubiquinol-cytochrome c reductase by a photoaffinity labeled Q derivative (8), and the stoichiometric binding between succinate-Q reductase and radioactive Q derivatives (9). The saturation behaviour of spin immobilization of a spin labeled Q derivative (10) by highly purified ubiquinol-cytochrome c reductase also suggests a specific protein:Q interaction in this segment of the electron transfer chain. This evidence,

Abbreviations used: PL, phospholipid; Q, ubiquinone; Q_0, 2,3-dimethoxy-6-methyl-1,4-benzoquinone; Q_1, 2,3-dimethoxy-6-methyl-5-(3-methylbutyl)-1,4-benzoquinone; 2-azido-Q_0, 2-azido-3-methoxy-6-methyl-1,4-benzoquinone; $Q_0C_{10}Br$, 2,3-dimethoxy-6-methyl-5-(10-bromodecyl)-1,4-benzoquinone; Q_2, 2,3-dimethoxy-5-geranyl-6-methyl-1,4-benzoquinone; 2-azido-Q_2, 2-azido-3-methoxy-5-geranyl-6-methyl-1,4-benzoquinone; 3-azido-Q_2, 3-azido-2-methoxy-5-geranyl-6-methyl-1,4-benzoquinone; 6-azido-Q_0C_{10}, 6-azido-5-decyl-2,3-dimethoxy-1,4-benzoquinone.

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although convincing, is derived mainly from Q derivatives with reporting groups located on the alkyl side chain of the Q molecule, the requirements for which have been shown to be less specific (1) than for substituents located on the benzoquinone ring. Similar studies with active Q derivatives bearing reporting or labeling groups on the benzoquinone ring should be more specific and might provide more information about the environment of the electron transferring site, since the redox reaction takes place on the benzoquinone ring. To this end, we synthesized a series of azido-Q derivatives with an azido group located on the benzoquinone ring and studied their interaction with Q- and phospholipid-depleted succinate-cytochrome c reductase. In the absence of light, 2-azido-3-methoxy-5-geranyl-6-methyl-1,4-benzoquinone (2-azido-Q_2), 3-azido-2-methoxy-5-geranyl-6-methyl-1,4-benzoquinone (3-azido-Q_2), and 6-azido-5-decyl-2,3-dimethoxy-1,4-benzoquinone (6-azido-Q_0C_{10}) show a partial restoration of succinate-cytochrome c reductase activity of the Q- and phospholipid (PL)-depleted preparation. The restored activity was sensitive to illumination with long wavelength UV light. However, great differences in the inhibitory effects of these Q derivatives were observed after photolysis, indicating that the binding of the benzoquinone ring of the Q molecule to protein is very specific. A small change in the arrangement of substitutents on the ring produced a pronounced effect on the binding behaviour and enzymatic activity.

In this communication we report the interaction and enzymatic activity of synthetic azido Q derivatives in succinate-cytochrome c reductase.

MATERIALS AND METHODS

Succinate-cytochrome c reductase (11) and its Q- and PL-depleted preparation (12) were prepared as previously described. The Q- and PL-depleted enzyme was finally dissolved in 50 mM phosphate buffer, pH 7.4 containing 20% glycerol and frozen at - 80° in small aliquots. Under these conditions, the enzyme is stable for several weeks.

For determination of electron transferring activity of synthetic Q derivatives the Q- and PL depleted enzyme was diluted to approximately 1 mg per ml with the same phosphate-glycerol buffer, pH 7.4. The diluted enzyme was mixed with various concentrations of Q derivatives before the addition of PL (asolectin) Stock solutions of Q derivatives were in 95% ethanol. Caution was excerised so that final concentrations of ethanol in the mixture did not exceed 2% because the depleted enzyme is sensitive to higher concentrations of ethanol. Since all the azido-Q derivatives are light sensitive, the whole sequence of preparation was performed in the dark unless otherwise specified.

Table I. Comparison of the Effectiveness of Azido-ubiquinone Derivatives in Electron Transfer Reaction in Succinate-Cytochrome c Reductase.

Addition	Concentration Used	Relative Activity
	nmoles/mg protein	%
$Q_0C_{10}Br$	20	100
Q_2	20	80
2-azido-Q_0	75	13
2-azido-Q_2	75	18
3-azido-Q_2	75	9
6-azido-Q_0C_{10}	75	16

Illumination of samples after addition of azido-Q derivatives was accomplished with a long wavelength UV lamp (model UV SL-25). The samples, 0.3 ml or less, were placed in quartz cuvettes (2 x 9 x 44 mm) and immersed in water in a quartz windowed Dewar; the temperature was kept at 2-5°. The distance between sample cuvette and lamp was about 5 cm.

Ubiquinone derivatives, 2,3-dimethoxy-6-methyl-5-(10-bromodecyl)-1,4-benzoquinone ($Q_0C_{10}Br$) and 2,3-dimethoxy-5-geranyl-6-methyl-1,4-benzoquinone (Q_2) were synthesized according to methods previously described (13). 2-azido-Q_2 (for structure, see Fig. 1) was synthesized from 4-methoxy-3-nitro-toluene via steps of reduction, oxidation, alkylation and nucleophilic substitution; 6-azido-Q_0C_{10} from 2,3-dimethoxy-phenol via steps of diazotization, reduction, oxidation, radical coupling alkylation and nucleophilic substitution. Details of these synthetic procedures will be reported elsewhere.

RESULTS AND DISCUSSIONS

Biological Activity of Synthesized Azido-Ubiquinone Derivatives-- Table I compares the biological activity of azido-Q derivatives in restoring electron transfer activity to Q- and PL-depleted succinate-cytochrome c reductase. The restoration reaction was carried out in the dark to avoid photoactivation of the azido group to active nitrene. The 2-azido-Q_2 restores about 18% of the electron transfer activity of succinate-cytochrome c reductase compared with the most effective Q derivative, $Q_0C_{10}Br$. Under the same conditions, restoration efficiency of Q_2 is about 80%. No significant difference in the efficiency of restoring activity is observed when the azido group is located on the 6 position of the benzoquinone ring. The 6-azido-Q_0C_{10} (see Fig. 1 for structure) has an efficiency of 16%. If the azido group is located in the 3 position of the

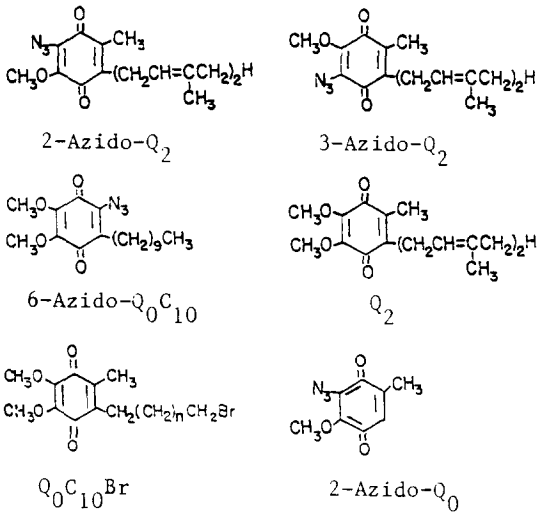

Fig. 1. Structure of Azido-Ubiquinone Derivatives

benzoquinone, a much lower activity is observed; only half of the activity of the 2-isomer. It is of interest to note that partial enzymatic activity also results from the addition of 2-azido-3-methoxy-6-methyl-1,4-benzoquinone (2-azido-Q_0), which has no long-chain alkyl group. The corresponding compound, 2,3-dimethoxy-6-methyl-1,4-benzoquinone (Q_0), is known to be absolutely inactive This result indicates that 2-azido-Q_0 binds to protein more tightly than does Q_0. In fact, the behavior of 2-azido-Q_0 is more similar to 2,3-dimethoxy-6-methyl-5-(3-methylbutyl)-1,4-benzoquinone (Q_1) than Q_0.

At the same concentrations, azido-Q derivatives showed little effect on intact succinate-cytochrome c reductase. This indicates that the binding of these Q derivatives to succinate-cytochrome c reductase is not as strong as that of native Q_{10} and the exchange between the bound Q_{10} and azido-Q derivatives is very slow or non-existent (14). If an equilibrium between bound Q and azido-Q derivatives does exist, one would expect to observe a decrease in activity, since the efficiency of azido-Q derivatives is much less than that of the native Q_{10}. Since Q_{10} is bound to protein more strongly than are azido-Q derivatives, if the azido-Q derivatives are first bound to enzyme, at least a partial replacement with $Q_0C_{10}Br$ is expected. Indeed, this deduction is substantiated by the result illustrated in Fig. 2, which shows the partial replacement of azido-Q derivatives by $Q_0C_{10}Br$. The Q- and PL-depleted succinate-

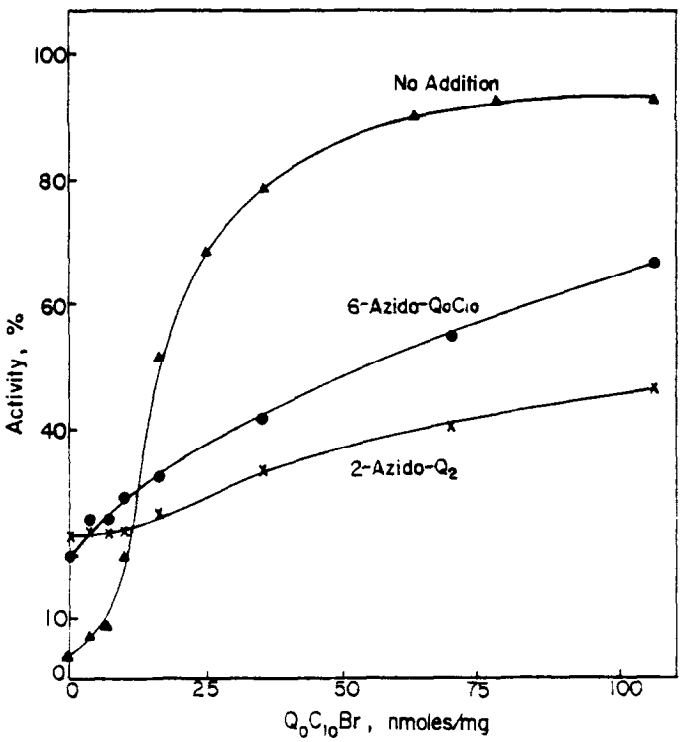

Fig. 2. Competition between $Q_0C_{10}Br$ and azido-ubiquinone derivatives for the binding site in succinate-cytochrome c reductase. one hundred and twenty µl aliguots of the Q- and PL-depleted succinate-cytochrome c reductase, 1 mg/ml in 50 mM phosphate buffer, pH 7.4 containing 20% glycerol, were mixed with 15 nmoles azido-Q derivatives before the addition of indicated amount of $Q_0C_{10}Br$ and 10 µl of phospholipid (asolectin, 20 mg/ml). The enzymatic activity was assayed after 50 min incubation at 0°. The 100% activity is equal to 4 µmoles succinate oxidized per min per mg protein at 25°.

cytochrome c reductase is first treated with 2- or 6-azido-Q derivative at 100 nmoles/mg protein, then subjected to titration with $Q_0C_{10}Br$. Activity increases as the concentration of $Q_0C_{10}Br$ is increased, indicating replacement of azido-Q derivatives by $Q_0C_{10}Br$, because the latter has higher activity. For example, in the case of 2-azido-Q_2, the activity at 100 nmoles $Q_0C_{10}Br$ per mg protein is found to be 46%, indicating that 31% of the binding sites have been replaced by $Q_0C_{10}Br$. Similarily, in the case of 6-azido-Q_0C_{10}, a 66% activity at 100 nmoles $Q_0C_{10}Br$ per mg protein represents a replacement of 59% of the sites of 6-azido-Q_0C_{10} by $Q_0C_{10}Br$.

Inhibitory Effect of Azido-Ubiquinone Derivatives on Succinate-Cytochrome c Reductase after Photolysis-- The advantage of using azido-Q derivatives is the ability of the azido group to be photoactivated to a nitrene which reacts

Table II. Effect of Photolysis on the Azido-Ubiquinone Derivative Restored Succinate-Cytochrome c Reductase Activity.

Q Derivatives	Relative Activity (%)		
	Before Illumination	After Illumination	Reactivated by $Q_0C_{10}Br$ after Illumination
2-azido-Q_0	13	5	64
2-azido-Q_2	18	0	2
3-azido-Q_2	9	0	11
6-azido-Q_0C_{10}	16	2	37
$Q_0C_{10}Br$	100	98	95

to form a covalent bond between the protein and Q. In this manner, a better definition of specific interaction can be obtained. If the azido-Q derivatives are bound to the same site as native Q, then the covalently linked azido-Q derivative:protein would prevent the further activation by addition of Q derivative or cause inhibition. The electron transfer reaction may involve delocalization of the benzoquinone ring of the Q molecule. When the azido group on the benzoquinone ring is covalently bound to protein after photolysis no free rotation or delocalization is possible. Therefore, a sharp loss of activity and irreversible inhibition is expected.

Table II shows the effect of photolysis on the restored succinate-cytochrome c activity by azido-Q derivatives. Photolysis of 2-azido-Q_2 reactivated succinate-cytochrome c reductase causes a loss of the activated activity and the ability to be restored by other Q derivatives. Similar results are observed in the case of 3-azido-Q_2 and 6-azido-Q_0C_{10}, although to a lesser extent. These results indicate that in the absence of native Q, 2-azido-Q_2 occupied fully the Q-binding site of the reductase. The partial activity expressed by the 2-azido-Q_2 in the dark is due to low efficiency of this Q derivative rather than low binding capacity because the photolyzed sample is almost totally inactive upon the addition of excess $Q_0C_{10}Br$. If the 2-azido-Q_2 only partly occupied the Q binding site, at least a partial reactivation would result after treatment with $Q_0C_{10}Br$, known to be the best Q derivative for monitoring electron transfer in

succinate-cytochrome c reductase. In the case of 6-azido-Q_0C_{10}, the binding may not be as specific as that of 2-azido-Q_2 because partial restoration of activity can occur after photolysis. It should be emphasized that the inhibitory effect of 2-azido-Q_2 is not due to the photolyzed product of 2-azido-Q_2 but to the formation of a covalent linkage between the Q derivative and protein. When 2-azido-Q_2 is photolyzed in ethanol solution before being added to the depleted enzyme, no inhibition is observed and the residual activity is no longer sensitive to photolysis. Upon addition of $Q_0C_{10}Br$ to the photolyzed 2-azido-Q_2 treated sample, more than 75% of maximal activity is restored. This result indicates that photolyzed 2-azido-Q_2 is unable to form a covalent linkage with the protein and is easily replaced by $Q_0C_{10}Br$.

Since photoactivated nitrene is known to be more reactive with the tyrosyl residues than other polypeptide amino acid residues (15), it seems likely that a tyrosyl group may be present in the quinone ring binding site. The difference in behaviour between 2-azido-Q_2 and 6-azido-Q_0C_{10} further suggests that the quinone binding site is very specific with respect to the substitutents on the benzoquinone ring. The lower inhibitory effect of 6-azido-Q_0C_{10} may be due to the fact that the photoactivated nitrene is some distance away from the tyrosyl group and thus may react non-specifically with other amino acid residues. Direct evidence for this deduction must await the identification of the photolysis product(s). Investigation toward this end is in progress in our laboratory.

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REFERENCES

1. Crane, F. L. (1977) Ann. Rev. Biochem., 46, 439-469.
2. Trumpower, B. L. (1981) J. Bioenerg. and Biomembrane, 13, 1-24.
3. Yu, C. A., and Yu, L. (1980) Biochemistry, 19, 3579-3585.
4. Yu, C. A., and Yu, L. (1981) Biochim. Biophys. Acta, in press.
5. Hatefi, Y., and Galante, Y. M. (1980) J. Biol. Chem., 258, 5530-5537.
6. Ackrell, B. A. C., Bethany-Ball, M., and Kearney, E. B. (1980) J. Biol. Chem. 2761-2769.
7. Vinogradov, A. D., Gavrikov, V. G., and Gavrikova, E. V. (1980) Biochim. Biophys. Acta, 592, 13-27.
8. Yu, C. A., and Yu, L. (1980) Biochem. Biophys. Res. Commun., 96, 286-292.

9. Yu, C. A., and Yu, L. (1982) J. Biol. Chem., in press.
10. Yu, C. A., and Yu, L. (1981) Biochem. Biophys. Res. Commun., 98, 1063-1069.
11. Yu, C. A., and Yu, L. (1980) Biochim. Biophys. Acta, 591, 409-420.
12. Yu, C. A., and Yu, L. (1980) Biochemistry, 19, 5715-5720.
13. Yu, C. A., and Yu, L. (1981) in "Function of Quinones in Energy Conserving Systems" (Trumpower, B. L., ed.,) Academic Press, New York , in press.
14. Schelhorn, J. E., Bustamante, P. L., and Loach, P. A. (1980) 5th Internationa Congress on Photosynthesis, Greece.
15. Chowdhry, V., and Westheimer, F. H. (1979) Ann. Rev. Biochem., 48, 293-325.