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Inhibition of succinate-ubiquinone reductase by nitrosalicyl-*N*-alkylamides

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The abilities of eight nitrosalicyl-*N*-alkylamides to inhibit succinate–ubiquinone reductase were compared. These compounds possess both a nitro group at 3- or 5-position of the benzyl ring, and a methyl, decyl, dodecyl or tetradecyl group substituted for the amide hydrogen of salicylamide. The result shows the following: (a) 3-Nitrosalicyl-*N*-alkylamides are stronger inhibitors than 5-nitrosalicyl-*N*-alkylamides when they have the same alkyl group. (b) The inhibitory abilities of both 3-and 5-nitrosalicyl-*N*-alkylamides increase with increasing alkyl group length. (c) Kinetic data show that the inhibition by 3-nitrosalicyl-*N*-tetradecylamide is noncompetitive with respect to the electron acceptor Q_2.

Introduction

3-Nitrosalicyl-*N*-alkylamides with alkyl groups of 4 to 18 carbon atoms were first synthesized as antimycin A analogues by Deckie et al. [1] and shown to have an antimycin-like inhibitory effect on Q_2H_2–cytochrome *c* activity in submitochondrial particles. Neft et al. [2] compared the inhibitory properties of substituted salicyl-*N*-(*n*-octadecyl)amides on the same activity. Later, 3-nitrosalicyl-*N*-methylamide was synthesized in this laboratory. Unlike Deckie's derivatives, 3-nitrosalicyl-*N*-methylamide acted like TTFA instead of antimycin A [3]. Based on this finding, we began to examine the nitrosalicylamides using another approach, hoping to verify the suspicion that long-chain nitrosalicylamides will act like both TTFA and antimycin A. In recent experiments we have found this to be true. By using succinate–cytochrome *c* reductase we found that 3-nitrosalicyl-*N*-methylamide inhibits electron transfer from succinate to DCIP [3], while nitrosalicylamides with an alkyl group longer than ten carbons inhibit both the electron transfer from succinate to DCIP and that from Q_2H_2 to cytochrome *c* (unpublished data). The latter result raises the question of whether there is a special site in succinate–cytochrome *c* reductase which affects both reactions or whether the inhibitors interact with the enzyme at two separate sites. We compared the inhibitory intensity and the distribution among phospholipids and water of 3-nitrosalicyl-*N*-alkylamides having different alkyl groups and found both of them changing in correlation with increasing length of their alkyl groups. The smallest derivative, 3-nitrosalicyl-*N*-methylamide, distributes chiefly in water phase, while the derivatives with alkyl chains longer than ten carbons distribute mostly in lipid phase (unpublished data). According to these data the site for 3-nitrosalicyl-*N*-methylamide inhibition should be located on the surface of the membrane, and the site for long-chain derivatives should be buried in the membrane. The question we raised above is still not solved by this kind of experiment. In order to understand the situation further it is desirable to split succinate–cytochrome *c* reductase into its components, succinate–ubiquinone reductase and QH_2–cytochrome *c* reductase, and examine how the inhibitor affects these two separated enzymes. If there are two separate inhibitory sites, both enzymes should be inhibited individually. We found this to be the case. Reported here are the inhibitory properties of nitrosalicylamide derivatives on succinate–ubiquinone reductase.

Abbrevations: Q_2, ubiquinone; Q_2H_2, ubiquinol; TTFA, 2-thenoyltrifluoroacetone; DCIP, 2,6-dicholoroindophenol; HMP, heart muscle preparation; SCR, succinate-cytochrome *c* reductase; SQR, succinate–ubiquinone reductase; QCR, Q_2H_2–cytochrome *c* reductase; SDH, succinate dehydrogenase; QPs, ubiquinone-binding protein in SQR.

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Materials and Methods

HMP was obtained from pig heart muscle using the purification procedure developed by King [4].The rate of oxygen uptake of HMP was determined to be about 500 μl/h per mg. Purification of SCR from HMP was also achieved by The method of King and co-workers [5]. Each milligram of protein contained about 2 nmol cytochrome *b* heme and 1 nmol cytochrome c_1. The activity was about 6 μmol cytochrome *c* reduction/min per mg. separation of SCR into SQR and QCR was realized by following procedure of Yu and Yu [6]. The activity of SQR was 4.7 μmol DCIP reduction/min per mg (no exogenous Q_2 added). The activity for catalysis of cytochrome *c* reduction by succinate was too low to be detected.

The enzyme activity assays for SCR [5], SQR [6] and the determination of cytochrome *b* and cytochrome c_1 content were carried out by the reported methods. Protein concentration for SCR was determined by the biuret method in the presence of H_2O_2 [7]. In the kinetic experiments, a series of concentrations of substrate (0.33–13.3 mM) and inhibitor (0–1.67 mM) were used. The total volume of the assay mixture was 3 ml.

Ubiquinone was synthesized in our laboratory according to Shunk [8]. Bio-Beads SM-2 were from Bio-Rad. Succinate was from BDH. DCIP was from Beijing Red Star Chemicals. All derivatives of salicylamide were synthesized in this laboratory according to the methods of Deckie [1] and Neft [2].

Results

Inhibitory abilities of 3-nitrosalicyl-N-alkylamides on purified SQR

As shown in Fig. 1, the inhibitory ability of 3-nitrosalicyl-*N*-alkylamides increases remarkably as the number of carbon atoms of the alkyl groups increases from 1 to 14. This indicates the hydrophobic nature of the environment around the inhibitory site of the enzyme. Similar changes were observed with 5-nitrosalicyl-*N*-alkylamides.

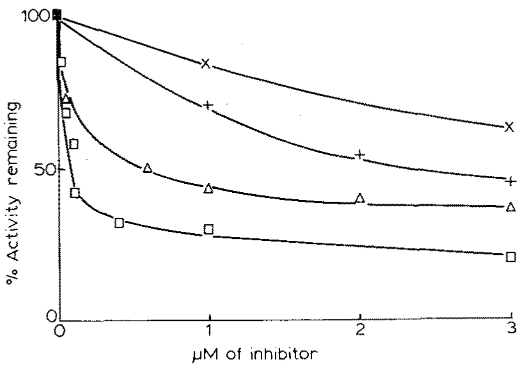

Fig. 1. Inhibition of SQR by 3-nitrosalicyl-*N*-alkylamides with different alkyl groups: ×, methyl; +, decyl; Δ, dodecyl; □, tetradecyl. 1 ml assay mixture contained 0.1 M phosphate buffer (pH 7.4), 20 mM succinate, 0.3 mM EDTA, O.O53 mM DCIP and the indicated amount of inhibitor. 5 μl SQR (0.54 mg/ml) was added to start the reaction.

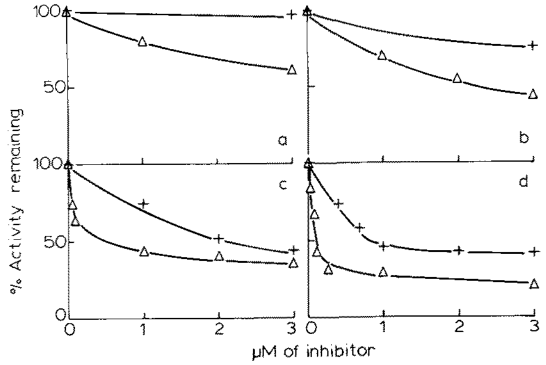

Fig. 2. Inhibition of SQR by 3- and 5-nitrosalicyl-*N*-alkylamides with different alkyl groups: (a) methyl; (b) decyl; (c) dodecyl; (d) tetradecyl. The curve Δ represents the inhibitor with nitro group at the 3-position and + at the 5-position. Conditions are the same as for Fig. 1.

3-Nitrosalicyl-N-alkylamides are stronger inhibitors than 5-nitrosalicyl-N-alkylamides on SQR

Fig. 2 compared the inhibitory abilities of 3-nitrosalicyl-*N*-alkylamides and 5-nitrosalicyl-*N*-alkylamides. It is obvious that an inhibitor having a nitro group at the 3-position is always stronger than if it has one at the 5-position. This means the inhibitory site in SQR has a sensitivity different from that of the ring part of the nitrosalicylamides. Whether or not the site in QCR has sensitivity similar to that of 3- or 5-nitrosalicylamides is worth looking into. Therefore, we measured the reduction of cytochrome *c* by Q_2H_2, although SCR was used instead of QCR for a preliminary experiment (Fig. 3), the result showed a remarkable difference. In contrast to the case with SQR, no apparent difference of inhibi-

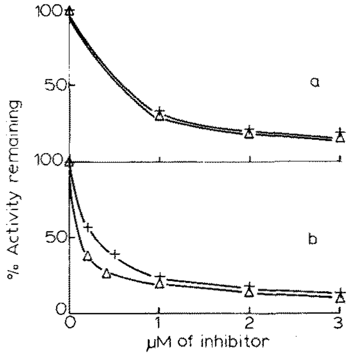

Fig. 3. Inhibition of QCR activity by 3- and 5-nitrosalicyl-*N*-alkylamides with (a) decyl and (b) tetradecyl as alkyl groups. The curve Δ represents the inhibitor with nitro group at the 3-position and + at the 5-position. 1 ml assay mixture contained 0.1 M phosphate buffer (pH 7.4), 0.3 mM EDTA, 0.1 mM cytochrome *c*, 50 μM Q_2H_2 and the indicated amount of inhibitor. 5 μl SCR (0.37 mg/ml protein) was added to start the reaction.

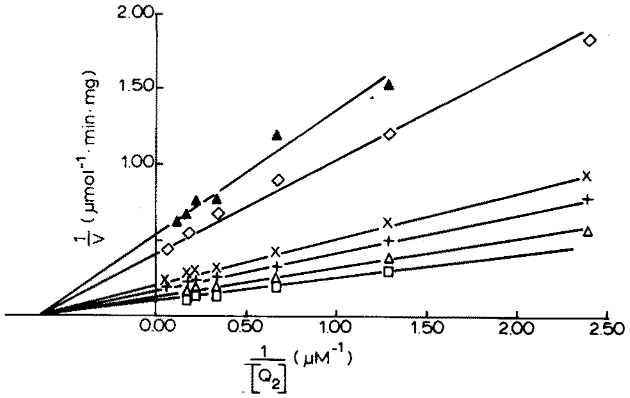

Fig. 4. Double-reciprocal plots of SQR inhibition by 3-nitrosalicyl-*N*-tetradecylamide. From top to bottom, the inhibitor concentrations were 1.667, 1.000, 0.333, 0.167, 0.033 and 0.000 μM, respectively. Conditions were the same as explained for Fig. 1, except that a 3 ml cuvette was used and Q_2 was added.

tory potency exists between the 3-position and the 5-position of substituted nitrosalicylamides. This might suggest that the inhibitions of SQR and QCR activities occur at two separate positions and these two sites have different properties.

The inhibition of SQR by 3-nitrosalicyl-N-tetradecylamide is noncompetitive with Q_2

The most powerful inhibitor of the eight derivatives, 3-nitrosalicyl-*N*-tetradecylamide was used to study the competitiveness/noncompetitiveness of the inhibition with respect to the direct electron acceptor, Q_2.

By the Michelis equation, we have:

$$V = \frac{V_{\max} \cdot ([S] + [Q_r])}{[S] + [Q_r] + K_m}$$

where $[Q_r]$ is the endogenous ubiquinone of the enzyme preparation, [S] is the concentration of the added Q_2. $[Q_r]$ was determined by curve fitting on the computer, with a series of known [S] and V. The calculated $[Q_r]$ was 0.055 μM. The K_m was calculated to be 1.4 μM. This value did not change when the concentration of inhibitor changed. $V_{\max}$ decreased with the increase of The concentration of inhibitor. This was expected for an inhibition of the noncompetitive type:

$$V_{\max} = \frac{V_0}{1 + [I]/K_i}$$

Fig. 4 is the double-reciprocal plot. The substrate concentration was corrected by adding 0.055 μM to the amount of Q_2 added. $K_m = 1.4$ μM was calculated from the intercept at the x axis. The intercept at the y axis increased as the concentration of inhibitor increased, as would be expected for a noncompetitive inhibition:

$$\text{intercept} = \frac{1 + [I]/K_i}{V_0}$$

By plotting the intercept versus [I] (Fig. 5), $V_0 = 9.1$ μmol/mg per min and $K_i = 0.42$ μM can be obtained.

The noncompetitiveness of the inhibition suggests that the inhibitor does not interact with the enzyme at the same site as the substrate Q_2 does.

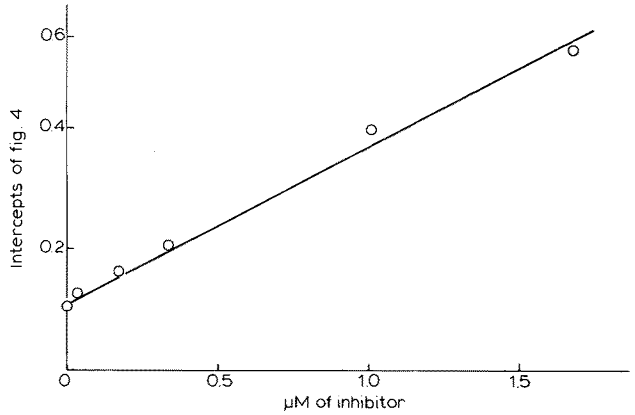

Fig. 5. Replot of Fig. 4, The intercepts versus the concentration of inhibitor.

Discussion

Although these nitrosalicylamides are structurally related to antimycin A, their properties are different. Antimycin A inhibits only electron transfer from ubiquinol to cytochrome *c*, but long-chain nitrosalicylamides inhibit both that from ubiquinol to cytochrome *c* and from succinate to DCIP. The latter property is similar to TTFA, but unlike 3-nitrosalicyl-*N*-tetradecylamide, TTFA inhibition is competitive with respect to the direct substrate O_2 according to Yu's data [9]. The site at which the present inhibitor, 3-nitrosalicyl-*N*-tetradecylamide, acts on the enzyme must be different from those of antimycin A and TTFA. The fact that both separated SQR and QCR can be inhibited by long-chain nitrosalicylamides means that each enzyme has its own inhibitory site. The difference in sensitivity to the change of the inhibitor's nitro group from the 3-position to the 5-position reveals the difference of the two inhibitory sites. Although the site on QCR has no obvious preference (the Fig. 3 experiment was done with SCR and the reduction of cytochrome *c* by QH_2 was measured), the site in SQR is much more sensitive to inhibitors having a 3-nitro group than those having a 5-nitro group. With these results, it seems that owing to the functional relatedness, the two sites have some common properties, but they are located in different environment, so that they differ in certain aspects.

In 1977, King et al succeeded in purifying the ubiquinone-binding protein QPs from the bc_1 particle [10,11]. By reconstituting with this QPs, the formerly inactive SDH became reactive in catalyzing the reduction of ubiquinone by succinate [9]. This result suggested that QPs is a component of SQR which is responsible for ubiquinone reduction. In our SQR preparation and those of many other authors, a considerable amount of cytochrome *b*-560 does exist [12–14]. At the present stage, the function of this cytochrome is still not clear. It may play some role in the electron flow process as supported by Hatefi's experiment [13]. Although it is difficult to measure the reduction of cytochrome *b*-560 by succinate, cytochrome *b*-560 reduced by dithionite is easily oxidized by fumarate or by ubiquinone in anaerobic condition [13]. Whether or not cytochrome *b*-560 takes part in the reduction of ubiquinone catalyzed by SQR is well worth looking into. Being a hydrophobic protein, cytochrome *b*-560 might act as an anchor in holding the SDH on the membrane [15]. Fig. 1 shows that the more hydrophobic an inhibitor the stronger its inhibitory ability. Since cytochrome *b*-560 is the only hydrophobic protein in SQR,it might be the position at which nitrosalicylamides act.

3-Nitrosalicyl-*N*-alkylamides are antimycin-like inhibitors according to the original work done by Deckie et al. [1]. 3-Nitrosalicyl-*N*-alkylamide binds to an 11 kDa polypeptide on QCR. This conclusion was obtainded by Wilson et al. [16] by using the analogue 3-azidosalicyl-*N*-(*n*-octadecyl)amide to label the antimycin binding site of QCR prepared from *Rhodopseudomonas* sphaeroides. Now we found that the same compound which Deckie studied has another inhibitory site – on SQR. A similar photolabeling experiment is already in progress with SQR in our laboratory. A question is whether there is any interaction between these two inhibitory sites. The inhibition of 3-nitrosalicyl-*N*-decylamide on the activity of electron transfer from succinate to DCIP is always stronger in SCR than in separated SQR and QCR (data not shown). This fact may indicate that there are interactions between the two sites.

By observing the noncompetitiveness of the inhibition, we have ruled out one possibility but leave many possibilities open. Ubiquinone reduction is achieved via a two-step procedure with ubisemiquinone as intermediate. The inhibitor 3-nitrosalicyl-*N*-alkylamide may affect only the second step. The inhibitor could compete with semiquinone or ubiquinol but not ubiquinone. The details are still worth further investigation.

Acknowledgement

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