ANTIBIOTIC EFFICIENCY OF NATURAL COUMARINS. XI.* ON THE MODE OF BINDING OF NATURAL COUMARINS TO THE HEART-MUSCLE MITOCHONDRIAL FRAGMENTS

V. DADÁK, P. ZBOŘIL and L. ZAHRADNÍČEK

Department of Biochemistry, Purkyně University, 611 37 Brno

Dedicated to Prof. Dr A. Okáč, on the occasion of his 70th birthday.

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The sulfhydryl groups were estimated in a preparation of succinic oxidase according to Keilin and Hartree, bovine serum albumin and several low-molecular mercapto compounds. The possibility of their reaction with coumarin derivatives inhibiting succinic dehydrogenase was eliminated. The binding and efficiency of coumarins toward the enzyme preparation are mediated by hydrophobic interactions *via* the lipophilic substituent of the coumarin molecule.

In the previous communication we described the inhibition of succinic oxidase by natural coumarins, using a heart muscle preparation[1]. The relationship between structure and activity of coumarin derivatives showed the inhibition to be dependent on the number and position of oxygen-containing substituents in the coumarin molecule and on the length but not position of the isoprene chain. Absence of the lipophilic chain in the coumarin molecule was accompanied by a loss of inhibitory effect which supports its importance for the binding of coumarins to the enzyme. On the other hand, an effect of ostruthin (6-geranyl-7-hydroxycoumarin) was demonstrated even with succinic dehydrogenase which is a "sulfhydryl" enzyme; the inhibition could be partly relieved by mercapto compounds which points to the possible role of SH groups in the binding of ostruthin to the enzyme[2]. It was the aim of the present work to establish the type of binding playing a role in the inhibitory action of ostruthin on the oxidation of succinate in the heart-muscle preparation.

EXPERIMENTAL

Chemicals. Coumarin derivatives were prepared or obtained as before[1]. Ostruthin isolated by Späth from natural sources was a gift of Prof. F. Wessely (Vienna). Hexahydroostruthin was prepared according to Späth[3] by hydrogenation on palladium. 8 mg ostruthin were dissolved in 1 ml ethanol and hydrogenated in the presence of 18 mg 5% Pd on barium sulfate. The consumption of hydrogen amounted to 3·3 equivalents and corresponds to the reduction of the double

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bond between carbons 3 and 4 of the lactone ring, and of two double bonds in the geranyl chain. When reducing with potassium borohydride, a $4 \cdot 10^{-5}$M alcoholic solution of ostruthin was reduced by adding 10 µl freshly prepared aqueous solution of KBH_4 (5 mg/ml). Other preparations were from commercial sources and were of an analytical grade.

Preparation of succinic oxidase from pig heart was obtained by a modification of the method of Keilin and Hartree as described by Slater[4]. The heart of a freshly killed animal is cooled with ice, the fatty tissue and integuments are removed and the tissue is ground in a meat grinder. The material is washed eight times with 10 litres tap water and is always thoroughly separated from the washings with gauze. The ground material is homogenized in a mixer with 1 liter 0·02M phosphate buffer of pH 7·4 for 3 min and the homogenate is centrifuged for 20 min at 800 g in a refrigerated centrifuge. The turbid supernatant fluid is filtered through gauze and stored, the sediment is diluted with 300 ml of the same buffer and recentrifuged. The sediment is discarded and the combined supernatants are centrifuged in the cold for 20 min at 25000 *g*. The supernatant is discarded and the sediment is mixed in a Potter-Elvehjem homogenizer with 30 ml 0·1M phosphate buffer of pH 7·4. The preparation obtained contains 25—40 mg protein/ml and is stored for 1 week at +3°C. The protein content was determined by a modified biuret method[5]. Urease was a commercial product of Squibb, New York. The activity of succinic dehydrogenase was determined spectrophotometrically with potassium ferricyanide at 420 nm ($\varepsilon = 1030$ liter mol^{-1} cm^{-1}) in a Beckman DU spectrophotometer. The dependence of the absorbance decrease on enzyme concentration was linear between 0·11 and 1·8 mg protein. Urease activity was determined both by the microdiffusion technique in Conway's unit cells and by a direct titration of released ammonia in the reaction mixture to methyl red. Coumarin derivatives were added to the reaction mixture in an ethanol solution. 3·3% ethanol did not decrease the activity of both enzymes.

The sulfhydryl groups were estimated photometrically according to Boyer[6] at 250 nm, further by potentiometric titration and on the basis of a reaction of SH groups with *o*-phthaldialdehyde according to Jocelyn and Kamminga[7]. Multoscope V was used as potentiometer for mercurimetric and argentometric estimation of the SH groups. The indication electrodes were of platinum and of silver. The cysteine electrode was prepared according to Cecil and McPhee[8]. A saturated calomel electrode was used as reference.

For following the binding of coumarins to mitochondrial fragments, the strong fluorescence of the derivatives of 7-hydroxycoumarin was employed[9], this being directly proportional to concentration in the range used (up to $2 \cdot 10^{-6}$M). After removing proteins from the reaction mixture, the drop of fluorescence in the reaction mixture was used for estimating the amount of derivative bound to the protein. Fluorimetric measurements were carried out in a Beckmann DU spectrophotometer with a fluorimetric attachment. The source of the primary radiation was a low-pressure mercury lamp with the maximum of emission at 360 nm, using a Schott UG 11 filter absorbing visible light. The absorption spectra were recorded in the same apparatus at 5 nm intervals or in a Unicam SP 700 spectrophotometer.

RESULTS

Determination of SH *groups.* The spectrophotometric determination of SH groups by titration with *p*-chloromercuribenzoate (PCMB) according to Boyer[6] was readily applicable to low-molecular sulfhydryl compounds. Titration of mercaptoethanol is shown in Fig. 1. A similar pattern was obtained for the titration of SH

groups of cysteine, glutathione and 2,3-dimercaptopropanol. Incubation with ostruthin and with other derivatives of coumarin had no effect on the consumption of PCMB. In a mixture with coumarins no decrease of the SH groups was found. Using a heart muscle preparation, the photometric estimation of SH groups when incubating in a 0·3M phosphate buffer of pH 7·3 gave no satisfactory results. After incubation with PCMB for 30, 60 and 90 min, the equivalence points could not be determined with accuracy. In 0·33M acetate buffer of pH 4·6 a 30 min incubation was sufficient and the values were well reproducible. The consumption of PCMB amounted to 80 nmol/mg protein in a fresh preparation and it dropped to 60 nmol PCMB/mg enzyme protein after a week of storage. Preincubation of the enzyme with ostruthin resulted in a slight decrease of PCMB consumption. The significance of the drop of SH group content in the presence of ostruthin is diminished by the turbidity

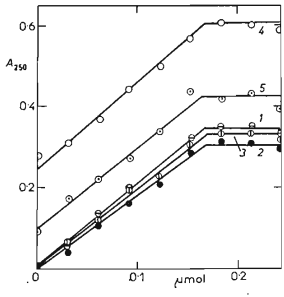

FIG. 1

Photometric Estimation of Sulfhydryl Groups of Mercaptoethanol in the Presence of Coumarin Derivatives

1·5 mM 2-mercaptoethanol with 0·75 mM coumarin derivative was incubated for 30 min at room temperature. After a tenfold dilution, the free sulfhydryl groups were estimated by titration with PCMB and the increase of absorbance was measured at 250 nm. The reaction medium contained in 3 ml: 1 ml of the mixture of mercaptoethanol with coumarin, 0·33M acetate buffer (pH 4·6) and PCMB as shown (in μmol). *1* No coumarin derivative *2* coumarin; *3* 4-hydroxycoumarin; *4* 7,8-dihydroxycoumarin (daphnetin); *5* ostruthin.

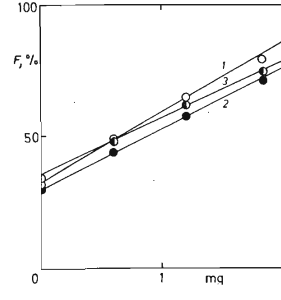

FIG. 2

Fluorimetric Estimation of Sulfhydryl Groups of Bovine Serum Albumin in the Presence of Coumarin Derivatives

0·5 ml solution of bovine serum albumin (mg) was incubated for 10 min at room temperature with 0·1 ml 3 mM coumarin or 4-hydroxycoumarin. Two ml of a mixture of 5% $HClO_4$ (neutralized with NaOH) containing 30 μM EDTA, 1·5 ml 0·5M - Tris-HCl buffer of pH 8·3 and 0·1 ml 1% *o*-phthaldialdehyde in methanol was added. After 15 min fluorescence was measured at 424 nm. The intensity of fluorescence in the presence of 3 mg albumin was taken as 100%. *1* Control; *2* coumarin, *3* 4-hydroxycoumarin.

which is due to the enzyme itself as well as to ostruthin which could not be removed by centrifugation for 20 min at 25000 *g*.

Potentiometric and fluorimetric estimation of sulfhydryl groups. Mercurimetric titration of the SH groups of a heart-muscle preparation yielded a titration curve, the derivation of which gave the point of equivalence at about 34 nmol sublimate/mg protein. This did not change in the presence of ostruthin. Also during argentometric titration ostruthin had no effect on the point of equivalence. When using ovalbumin as a model protein, a 1-hour preincubation of ovalbumin with ostruthin had no effect on the results of mercurimetric or argentometric titration. The results of these experiments indicate that ostruthin does not react with protein SH groups. The possibility cannot be excluded, however, that heavy metal ions with high affinity for sulfhydryl groups displace ostruthin from its binding.

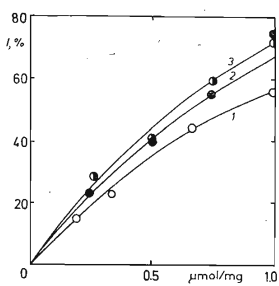

FIG. 3

Inhibitory Effect of Ostruthin and Its Hydrogenated Products on Succinic Dehydrogenase

The reaction mixture (3 ml) contained 0·07M phosphate buffer of pH 7·4, 50 μmol sodium succinate, 20 μmol potassium cyanide 4 μmol potassium ferricyanide and increasing concentrations of the coumarin derivative (μmol/mg). The reaction was initiated by adding 0·2 mg protein of the enzyme preparation and the decrease of ferricyanide was measured for 3 min at 22°C. *1* Ostruthin; *2* hexahydroostruthin; *3* ostruthin reduced with potassium borohydride.

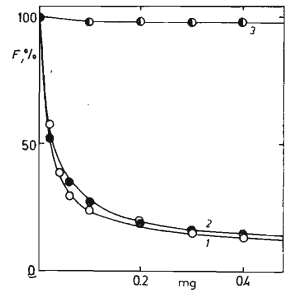

FIG. 4

Binding of Coumarin Derivatives to Mitochondrial Fragments

2 μM coumarin derivative was incubated for 30 min at room temperature in 0·1M-Tris HCl buffer of pH 7·4 with the enzyme preparation (mg). The enzyme preparation was then separated by a 20 min centrifugation at 25000 *g* and the fluorescence of the supernatant was measured at 460 nm. The fluorescence of the coumarin derivative incubated without the enzyme is taken as 100%. *1* Ostruthin; *2* ostruthin reduced with potassium borohydride; *3* β-methylumbelliferone.

The possibility of coumarin reacting with protein SH groups was further studied on the basis of fluorescence arising after the reaction of thiols with *o*-phthaldialdehyde[7]. Fig. 2 shows that preincubation with coumarin and the inhibitory 4-hydroxycoumarin affects only negligibly the fluorescence arising from the reaction between bovine serum albumin and *o*-phthaldialdehyde. As its intensity depends on the presence of free SH-groups and as compounds interfering with them (*e.g.* N-ethylmaleimide) suppress the fluorescence it appears that coumarin and 4-hydroxycoumarin do not react with the sulfhydryl groups of serum albumin. In this method we could not work with ostruthin or with umbelliferone since their intense fluorescence interfered with the estimation. When using a heart-muscle preparation, no linear relationship between fluorescence and the amount of preparation was obtained.

Binding of coumarins to mitochondrial fragments. Derivatives of coumarins as α,β-unsaturated carbonyl compounds offer the possibility of addition between the 3rd and the 4th carbon of the coumarin ring and the SH group. By saturating this bond the possibility of reaction with the SH groups is lost. We prepared here two hydrogenated derivatives of ostruthin and observed their inhibitory effect on succinic dehydrogenase in a heart-muscle preparation. In the case of blocking of SH groups of the enzyme with ostruthin they should not show any inhibition. Using palladium, we prepared hexahydroostruthin[3] with a saturated double bond between the C-atoms of the lactone ring and the saturated geranyl chain. Reduction with potassium borohydride results in a deeper hydrogenations as may be deduced from the absorption spectra of the compounds in the UV region. The inhibitory action of both hydrogenated products in comparison with ostruthin is shown in Fig. 3. It appears that the inhibitory effect is not due to the double bonds of the ostruthin molecule. Hydrogenated derivatives show an equal or even superior inhibitory effect. It follows from the results that the SH groups are not included in the inhibition and binding of ostruthin to the heart-muscle preparation. The results further confirm the postulate on the primary importance of the lipophilic chain where also the presence of unsaturated bonds for inhibitory activity is of no importance. Likewise, the phenolic hydroxyl plays no significant role in the inhibitory effect of ostruthin since its ionization or methylation do not decrease the inhibitory effect[1,2]. A similar conclusion holds for ammoresinol (3-farnesyl-4,7-dihydroxycoumarin).

A direct proof of the function of the isoprenic chain during binding to the enzyme preparation was provided by the experiment where the amount of ostruthin and of its derivatives bound after incubation to the heart-muscle preparation was investigated. We made use of the intense fluorescence of the derivatives of 7-hydroxycoumarin (umbelliferone)[9]. The intensity of fluorescence of ostruthin and of its hydrogenated product dropped markedly after removing the enzyme preparation from the mixture while the fluorescence of the related β-methylumbelliferone remained unchanged after incubation with the enzyme preparation. The drop of fluores-

cence of ostruthin and hexahydroostruthin depends on the amount of protein in the incubation mixture as follows from the dependence shown in Fig. 4. Here, too, it may be seen that the phenolic hydroxyl at carbon 7 of the coumarin derivative has practically no influence on the binding to the enzyme.

DISCUSSION

It follows from the results that the inhibitory activity of ostruthin on succinic oxidase requires the presence of a lipophilic side chain in the molecule, through which the interaction of ostruthin with the structure-bound enzyme takes place. The hydrophobic character of the interaction is indicated also by the slight inhibition of geraniol itself while 7-hydroxycoumarin has no effect on the enzyme[2]. The geranyl chain present in the ostruthin molecule shows apparently a high affinity for mitochondrial fragments. Fynn and Redfearn[10] examined the possibility of binding of ubiquinone homologues with different lengths of the isoprenic chain (Q-1, Q-2, Q-3, Q-5 and Q-10) to a similar preparation from heart muscle. The highest binding affinity for succinic oxidase was found with the Q-2 derivative, *i.e.* the homologue with a geranyl side chain.

The finding that binding to SH groups plays no role in the inhibitory effect of coumarin derivatives to succinic oxidase is in agreement with the earlier results obtained by titration with iodine[11]. Here too, incubation with ostruthin resulted in no decrease of the amount of free SH groups in cysteine, glutathione and other low-molecular mercapto compounds. The absorption spectra of a mixture of ostruthin with cysteine, mercaptoethanol or the heart-muscle preparation did not differ from the sum of spectra of the compounds alone. Similarly, the spectra of diacetylammoresinol and of other inhibitory coumarin derivatives did not change due to the presence of cysteine and mercaptoethanol. The results obtained are at variance with the previously observed reactivation effect of mercapto compounds on succinic dehydrogenase inhibited by ostruthin[2]. This might be explained by a reactivation of the SH groups of the enzyme inhibited by contact with oxygen. The effect of mercapto compounds on the enzyme activity was substracted during the estimation of reactivation but apparently, in the presence of a coumarin derivative, the pattern of enzyme inactivation in the reaction mixture, or its reactivation by mercapto compounds, is different. Another factor that might play a role in the observed reactivation effect is the possibility of a nonenzymic reduction of methylene blue by the mercapto-compounds.

The lack of participation of sulfhydryl groups in the binding of coumarin derivatives to enzymes has a more general validity. Ostruthin and other inhibitory coumarin derivatives do not inhibit the typical "sulfhydryl" enzyme, urease, whether the measurement is done by the micro-diffusion technique in Conway's unit cells or by titration directly in the reaction mixture. To exclude the possibility of a preferential

reaction of coumarins with non-essential SH groups of urease the enzyme activity was blocked approximately by 50% with PCMB. Under the influence of a number of coumarins tested no further increase of inhibition was observed. Hence it follows that even with urease no reaction between the SH groups and the coumarin derivatives takes place. Preliminary results indicate that solubilized succinic dehydrogenase containing essential SH groups is not inhibited by ostruthin.

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