STERIC AND ELECTRONIC EFFECTS ON THE UNCOUPLING ACTIVITY OF SUBSTITUTED 3,5 DICHLOROSALICYLANILIDES

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1. Introduction

The wide variety of structures found in compounds that uncouple oxidative phosphorylation and other energy-linked reactions from mitochondrial electron transfer has hindered attempts to deduce the mechanism of uncoupling from considerations of structure of uncoupler molecules. Recently, evidence has been presented that uncouplers bind to a specific site in the mitochondrial membrane which has a stoichiometry of approximately one site per cytochrome *a* [1,2]. The uncoupler 2-azido-4-nitrophenol has been reported to label preferentially in 40% yield a single mitochondrial polypeptide when the azide group of the uncoupler bound to this site is photoactivated [2]. Measurements of the pH dependence of the uncoupling activity of various uncouplers indicates they may act preferentially as acids or bases with the salicylanilides having greatest activity as the conjugate base monoanion [3]. In order to elucidate the steric and electronic requirements for uncoupling activity in this type of compound, we have prepared a series of salicylanilides and related compounds with systematic variation of structure. In this paper, we report that the anilides of the commercially available 3,5-dichlorosalicylic acid are potent uncouplers and that the uncoupling activity of these compounds has certain stringent steric requirements.

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2. Materials and methods

The 3,5-dichlorosalicylanilides were prepared by a procedure provided by Dr K. C. Tsou of the Harrison Department of Surgical Research, University of Pennsylvania School of Medicine (personal communication). A solution of 10 mmoles of 3,5-dichlorosalicyclic acid (Eastman Organic Chemicals) and 15 mmoles of the appropriate aniline (Aldrich Chemical Co.) in 20 ml toluene with 5 mmoles PCl_3 (Fisher Chemical Co.) were refluxed for one hr: the product was isolated by precipitation in a ten-fold vol of 1 M $NaHCO_3$, filtration, drying, and recrystallization from ethanol–acetone. The *p*-nitrophenyl ester and *p*-nitrophenylhydrazide of 3,5-dichlorosalicylic acid were prepared in like manner. Reaction of the *p*-nitrophenyl ester with concentrated NH_4OH in tetrahydrofuran yielded the 3,5-dichlorosalicylamide. IR spectra in KBr discs and NMR spectra in perdeuterated dimethylsulfoxide (DMSO) (Merck) were in full accord with the structures expected. The pKa values of the salicylanilides were determined in 20% and/or 70% DMSO by monitoring the increase in the absorbance band in the 350 to 360 nm region characteristic of the anion as a function of pH. Stock solutions of the uncouplers were prepared for assay in dimethylformamide (DMF).

Uncoupler activity was assayed by the increase in respiratory rate of rat liver mitochondria with succinate as substrate in the presence of rotenone, measured with the oxygen electrode as described by Estabrook [4]. The increase was shown to be essentially linear until the maximum respiratory rate was attained; this maximum activity was independent of uncoupler type

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[3]. The slope of the linear increase, which has the units (moles O_2) $(min)^{-1}$ (moles uncoupler)$^{-1}$ is converted to the more general units (moles X~I) (moles uncoupler–min)$^{-1}$. For the case of respiration with succinate in the presence of rotenone, it is assumed that four X~I are generated per O_2 consumed. This measure of uncoupling activity is effectively a turnover number for the discharge of the high energy intermediate (whose nature is here not specified). Concentrations of uncoupler used in this work were sufficiently low that the linear relation was obeyed. Details of the method are given in [1].

3. Results

The 3,5-dichlorosalicylanilides and structural analogues prepared in this study are listed in table 1, along with melting points, pKa values and uncoupling activity, expressed as (moles X~I) (moles uncoupler–min)$^{-1}$. With the exception of 3,5-dichlorosalicylamide, which is sparingly water soluble, all the compounds listed are high-melting crystalline solids, insoluble in both water and hydrocarbon solvents, but having some solubility at room temperature in both DMF and DMSO. The compounds comprising the 4′-substituted anilides show that uncoupling activity is present in molecules with electron releasing groups such as methoxy and methyl, as well as electron withdrawing groups such as chloro and nitro, at the para position of the aniline moiety. All these compounds show maximal uncoupling activity at pH 7.1, have very similar pKa values and have very similar uncoupling activities, with the exception of the 4′-chloro compound which is three-fold more active than the others. The change of activity with pH in this pH region is not very marked, as shown by the values at pH 7.4. Multiple substitution of the aniline moiety decreases the pKa somewhat, but has little effect on the uncoupling activity: the 2′ chloro-4′-nitro has essentially the same

Table 1
Properties and uncoupling activities of substituted 3,5-dichlorosalicylanilides and structural analogues

Substituents	m.p., °C.	pKa (20% DMSO)	(moles X~I) (moles uncoupler–min)$^{-1}$ pH=7.1	pH=7.4
4′-Methoxy	173–174	4.9	444	300
4′-Methyl	180–181	4.9	328	296
Unsubst.	124–125	4.7 4.8[a]	456	408
4′-Fluoro	162–163	4.8	380	364
4′-Chloro	190–191	4.7 4.8[a]	1230	1230
4′-Nitro	>330	4.6	480	480
2′-Chloro-4′-nitro	232–233	4.0[a]	400	–
2′,4′,5′-Trichloro	185–187	4.4[a]	1050	–
p-Nitrophenyl-[b] hydrazide	280 (dec.)	4.3[a]	40	38
p-Nitrophenyl[c] ester	236–237	approx 4.6[d]	22	23
Amide[e]	181–182	5.6	2.0	2.8

a) Value determined in 70% DMSO. b) Compound is 3,5-dichlorosalicyl-*p*-nitrophenylhydrazide. c) Compound is *p*-nitrophenyl-3,5-dichlorosalicylate. d) *p*-Nitrophenyl-3,5-dichlorosalicylate is labile towards hydrolysis in alkaline solutions, resulting in an uncertain endpoint. The pKa value is estimated by titration starting at low pH, assuming the same extinction for the anion as for the corresponding salicylanilide. e) Compound is 3,5-dichlorosalicylamide.

activity as the 4′-nitro derivative and the 2′,4′,5′-trichloro is but slightly less active than the 4′-chloro derivative. If, however, the anilide linkage is perturbed by replacing the –NH– group with –O– as in the *p*-nitrophenyl ester, or with the –NH–NH– group as in the *p*-nitrophenylhydrazide, the uncoupling activity drops by an order of magnitude. The pKa and the optimum pH range for uncoupling activity of these two compounds is nearly the same as for the salicylanilides. If the phenyl substituent on nitrogen is omitted, as in the amide, the uncoupling activity drops two orders of magnitude, the pKa is one unit higher, and the pH optimum for uncoupling activity shifts to pH 7.4.

4. Discussion

The most potent uncoupler of the group in table 1 is 3,5-dichloro-4′-chlorosalicylanilide. Its activity is quite insensitive to pH in the region of 6.8 to 7.4 where it is maximally active. It is somewhat less potent than S-13 (5-chloro-3-*tert*-butyl-2′chloro-4′-nitrosalicylanilide) at pH 7.1 and 7.4: the uncoupling activities of S-13 at these pH values are 4000 and 5000 (moles X~I) (moles uncoupler–min)$^{-1}$, respectively. It is more potent than S-6 (5-chloro-3-(*p*-chlorophenyl-4′-chlorosalicylanilide) whose uncoupling activities are 700 and 800 (moles X~I) (moles uncoupler–min)$^{-1}$ at pH 7.1 and 7.4, respectively. Both S-13 and S-6 have maximal activity at pH 7.9 with uncoupling activities of 7500 and 1700 (moles X~I) (moles uncoupler–min)$^{-1}$, respectively. But for the range of physiological pH, 3,5′dichloro-4′-chlorosalicylanilide, to which we give the abbreviation DCCA, is a useful member of the salicylanilide group of inhibitors because of both its high activity and ease of preparation from readily available starting materials.

It is also evident from table 1 that it is the substituents on the salicyclic acid moiety which primarily determine the pKa of the salicylanilide, as would be expected if the proton is lost from the phenolic hydroxyl group. With the 3,5′ dichloro substituents, the pKa values range between 4.6 and 4.9 for the 4′-monosubstituted salicylanilides. The pKa for the membrane-bound form of other uncouplers has been shown to shift from 1 to 2 units to a higher value by Wilson, et al. [1]. In these compounds, the pKa values are below 5.0, making them essentially all in the monoanion form in the physiologically interesting pH range, even when bound to the mitochondrial membrane. The 3′chloro substituent is apparently not quite bulky enough for optimal activity, however, since the bulkier 3-*tert*-butyl group in S-13 and 3-(*p*-chlorophenyl) group in S-6 enhance the uncoupling activity of these compounds over that observed with their 3′ chloro analogues. These differences in activity demonstrate the sensitivity of these uncouplers to steric effects, implying that they bind at a specific site with particular steric requirements. This is in agreement with the conclusions of Draber et al. [5] derived from an extensive study of the structural requirements of the carboxy cyanide group of uncouplers.

The uncoupling activity of the substituted 3,5′ dichlorosalicylanilides is relatively insensitive to the electron withdrawing or releasing properties of the substituents on the aniline moiety, contrary to the original hypothesis of Williamson and Metcalf [6] that electron withdrawing groups are required. A reasonable structure for a salicylanilide anion, the active uncoupling species [1], is shown in fig.1. While the substituents on the aniline moiety contribute relatively little to the stabilization or destabilization of the structure in fig.1, the aromatic ring itself appears necessary to maintain coplanarity of the salicylanilide anion structure through electron delocalization. The uncoupling activity of the salicylanilide appears to be very sensitive to changes which alter the structure of the anion: neither the phenyl ester nor the phenyl hydrazide can maintain the planar structure shown in fig.1, and these compounds have much reduced uncoupler activity. Hypotheses which emphasize nonspecific acid–base properties of an uncoupler or its enhancement of proton conductance across lipid membranes [7–11] would have predicted neither this difference nor the sensitivity of activity to steric effects of the 3-substituent in the salicylic acid moiety.

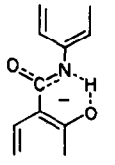

Fig.1. Structure of salicylanilide anion stabilized by electron delocalization and hydrogen bonding.

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