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DIFFERENTIAL EFFECTS OF DENATURING AGENTS ON ACETYLCHOLINESTERASE:

INSENSITIVITY OF THE REACTION OF METHANESULFONYL FLUORIDE COMPARED TO DIISOPROPYLPHOSPHOROFLUORIDATE AND *p*-NITROPHENYL ACETATE

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Summary

The effects of various agents that could be expected to perturb enzyme structure in a non-specific and reversible manner (alcohols, dimethylsulfoxide, dimethylformamide, dinitrobenzene, urea and guanidine · HCl) have been determined on reaction of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) with a substrate, *p*-nitrophenyl acetate, and two irreversible inhibitors, diisopropylphosphorofluoridate and methanesulfonyl fluoride. In all three of these reactions an acyl group (acetyl, phosphoryl or sulfonyl respectively) bonds covalently with the active center of the enzyme. As expected, the reactions of *p*-nitrophenyl acetate and diisopropylphosphorofluoridate were severely retarded by most of these agents. By contrast, reaction of methanesulfonyl fluoride was usually depressed to a far smaller degree, and in two cases was faster. These findings are of interest in connection with: (1) differing requirements for the integrity of the active center in catalysis with various substrate analogs, and (2) the mechanism by which cationic substrate analogs accelerate reaction of the enzyme with methanesulfonyl fluoride.

Introduction

The reaction of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) with methanesulfonyl fluoride has attracted great interest because it is accelerated in the presence of certain cationic substrate analogs, such as tetramethyl ammonium ion [1—5]. The active center of this enzyme contains two distinguishable regions: a specificity or "anionic" site that adsorbs substrate cations,

and a catalytic or "esteratic" site responsible for splitting the ester bond in the substrate. Methanesulfonyl fluoride is a pseudo-substrate that reacts near the anionic site, probably with an essential serine residue in the esteratic site, to form a sulfonylated enzyme [1,6,7]. True substrates undergo a completely analogous reaction, acetylating the same serine hydroxyl in the first step of their hydrolysis. What appears to be of special significance for our understanding of the enzyme mechanism, therefore, is that reaction of the catalytic site with methanesulfonyl fluoride, which is a small molecule comparable in size and reactivity to the acyl group of acetylcholine, is favored by the presence, in the adjoining specificity site, of an analog of choline, the alcohol residue making up the remainder of the acetylcholine molecule. In general, when binding of a small molecule to a protein increases the rate at which the latter reacts with a chemical reagent we may reasonably attribute the effect to a conformational change induced in the protein. In the present instance, we may add that this conformational change produced by the quarternary ammonium ion could play a role in modulating the activity of the catalytic site. An induced-fit mechanism could operate, involving a single active center, or an allosteric mechanism involving two or more sites of the multi-site, tetrameric enzyme [8—10]. Studies of this reaction could therefore be of great importance, provided however that the interpretations are not wholly misdirected; that is, provided such modifications of enzyme structure can be induced by acetylcholine itself and involve specific forces between adsorbed cations of particular structure and anionic sites in the enzyme. The word "specific" is used here to designate interactions that play a role in binding and catalysis with the natural substrate, and that promote catalytic efficiency; whereas "unspecific" interactions are those of certain analogs of the natural substrate, which oppose catalytic efficiency. An example of the latter is the effect of the enlargement of the acyl group, in butyrylcholine. This ester is more firmly anchored at the active center of erythrocyte acetylcholinesterase than is acetylcholine, by a factor of approximately 4, but it is not hydrolyzed at any significant rate. It follows that the binding forces between the butyryl group and the enzyme, though strong, are unproductive [11].

The idea that the acceleration of methanesulfonyl fluoride reaction by substrate analogs involves specific forces (in this sense) has been challenged, mainly because of two findings: (1) A wide variety of cationic structures accelerate sulfonylation, even large symmetrical ions such as tetrapentyl ammonium and tetraphenyl arsonium, which should partially overlap the esteratic site and obstruct entry of methanesulfonyl fluoride, were their centers of positive charge lined up at the same position as that of acetylcholine [12]. Such ions block reaction with substrates and with larger inhibitors like diisopropylphosphorofluoridate [13], and are therefore probably adsorbed adjacent to the anionic site. The binding, in such cases, must be non-specific. (2) Acetylcholine analogs having bulky alkyl substituents on the nitrogen atom react more slowly than normal substrates but accelerate the reaction with methanesulfonyl fluoride, which is to say that non-specific and disorienting interactions at the anionic site reduce rates of acetylation by substrates but increase rates of sulfonylation [12].

Here I wish to present additional evidence for the non-specific nature of

the forces involved in accelerating sulfonylation; to wit, that this reaction is relatively insensitive to many agents that should cause unspecific perturbations of enzyme structure, such as occur in reversible denaturation, and that in some cases these agents are themselves able to accelerate the rate.

Materials and Methods

Acetylcholinesterase from the electric organ of the electric eel and from bovine erythrocytes was obtained from Sigma Chemical Co. (types V and I respectively). All chemicals were of reagent grade.

Reactions were studied at pH 8.0, 26° C. During treatment of enzyme with methanesulfonyl fluoride or diisopropylphosphorofluoridate, activity in aliquots (0.05 ml) withdrawn at intervals from the reaction mixture was determined with acetylthiocholine, in a volume of 5 ml, by means of a modified Ellman assay [14,15]. The half times for inactivation were taken from plots of enzyme activity, on a log scale, against time, and these plots were linear. As half times, at a given inhibitor concentration, are inversely related to pseudo-first order inactivation rate constants, the ratio of inactivation rates in the presence and absence of denaturant could be calculated from the ratio of half times. Rates of *p*-nitrophenyl acetate hydrolysis under various conditions were determined directly from the absorbance of the product *p*-nitrophenol at 412 nm. A Beckman DB-G recording spectrophotometer equipped with a thermostatted cell compartment was employed for this purpose.

Experimental Results

Relative rates of enzyme reaction with methanesulfonyl fluoride, diisopropylphosphorofluoridate and a substrate, *p*-nitrophenyl acetate, were deter-

TABLE I

Relative rates of reaction of eel acetylcholinesterase with 0.018 mM diisopropylphosphorofluoridate (DFP), 1.0 mM *p*-nitrophenyl acetate (NPA) and 0.58 mM methanesulfonyl fluoride (MSF) in the presence of various agents. Temperature 26° C, pH 8.0, potassium phosphate buffer, 20 mM.

Agent	Concentration	Relative rates of reaction with acetylcholinesterase*		
		DFP	NPA	MSF
Ethanol	10%	0.1	0.24	0.7
n-Butanol	4%	0.2	0.16	1.15
t-Butanol	10%	0.1	0.05	0.8
Dimethylformamide	1%	0.05	0.24	0.8
Dimethylformamide	2%	0.05	0.14	0.7
Dimethylformamide	4%	0.00	0.12	0.7
Dimethylsulfoxide	1%	0.15	0.40	0.4
Dimethylsulfoxide	3%	0.00	0.17	0.4
Dimethylsulfoxide	6%	0.00	0.10	0.3
m-Dinitrobenzene	0.5 mM	0.2	0.25	0.9
Urea	1.6 M	0.8	0.8	0.8
Guanidine · HCl	0.3 M	0.8	0.6	2.7

* The reference rates, in the absence of agent, are set at 1.0.

TABLE II

Relative rates of reaction of bovine erythrocyte acetylcholinesterase with 0.009 mM diisopropylphosphorofluoridate (DFP), 1.0 mM *p*-nitrophenyl acetate (NPA) and 0.36 mM methanesulfonyl fluoride (MSF) in the presence of various agents. Temperature 26°C, pH 8.0, potassium phosphate buffer, 20 mM.

Agent	Concentration	Relative rates of reaction with acetylcholinesterase*		
		DFP	NPA	MSF
n-Butanol	4%	0.2	0.10	0.8
Dimethylformamide	3%	0.00	0.10	0.6
Dimethylsulfoxide	3%	0.00	0.19	0.25
m-Dinitrobenzene	0.5 mM	0.3	0.38	1.05
Urea	1.6 M	0.7	0.34	0.7
Guanidine · HCl	0.3 M	0.8	0.58	2.7

* The reference rates, in the absence of agent, are set at 1.0.

mined in the presence of a variety of denaturing agents (Tables I and II). The three reactions involve acylation of the enzyme by a neutral molecule and are therefore analogous. (The substrate is rate-limited at the acetylation rather than deacetylation step, as shown by the fact that its V is much lower than that of acetylcholine [12]). It is seen that methanesulfonyl fluoride reaction is generally less affected by these adverse conditions than the others. In two cases it is accelerated under conditions where the other reactions are retarded: with *n*-butanol (though only with eel enzyme) where the rate increase is slight, and with guanidine · HCl where it is large (with both enzymes).

Discussion

Enzyme perturbations in the presence of denaturing agents have here been shown to affect catalytic activity in different ways or to varying degrees, depending on the substrate or substrate analog employed. A possible obstacle in the interpretation of such experiments is the known stabilization of enzymes following substrate binding, but this difficulty is avoided if the experiments are carried out at substrate concentrations well below the half-saturation constants for the system [16]. That this condition applies to the experiments reported here is shown by the fact that the rates of reaction with all three substrate analogs, at the concentrations employed, were directly proportional to their concentrations. Further, the affinity constant for diisopropylphosphorofluoridate is reported to be 1.2 mM with bovine erythrocyte enzyme, pH 7.6 and 25°C [17]. The concentrations used in the present study were in the order of 100 times lower than this. The affinity constant for methanesulfonyl fluoride has not been determined exactly but was shown to be much greater than 3 mM with electric eel acetylcholinesterase, pH 7, 25°C [7], and 3 mM is about 10 times higher than the concentrations used here. K_M for *p*-nitrophenyl acetate with erythrocyte acetylcholinesterase at pH 7.5, 26°C, is 4 mM [11], which is 4 times the present concentration. Though not a requirement of the method, the rate-limiting step in reaction of all three is acylation, and all are neutral molecules which, unlike organic cations, would not be expected to stabilize the enzyme against denaturation. Thus, a wide variety of quarternary ammonium

ions were observed to protect acetylcholinesterase against thermal inactivation (Krupka, R.M., unpublished), whereas the enzyme is known to be adversely affected by the presence of neutral molecules such as the denaturing agents employed here (Tables I and II).

Indophenyl acetate [16] and possibly *n*-butanol (Table I) accelerate reaction of methanesulfonyl fluoride. This could be due to an increased tendency of the inhibitor to form a complex with the enzyme in their presence, or to faster reaction of this complex to give a sulfonyl enzyme derivative (unpublished results).

Acceleration due to guanidine · HCl, on the other hand, could be related to that by other cations bound at the anionic site, possibly involving a field effect of a positive charge near the leaving fluoride anion. Guanidine resembles organic cations which accelerate the reaction, and differs from metal ions which have a far smaller effect, in size as well as in affinity for a non-polar environment; and many observations have suggested a close association between the anionic site and lipophilic residues in the protein [11,18].

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