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Methanesulfonyl Fluoride: A Probe of Substrate Interactions in the Catalytic Site of Acetylcholinesterase

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Methanesulfonyl fluoride, which reacts at the catalytic site of acetylcholinesterase causing inactivation, is a useful probe of substrate binding. This is because molecules absorbed in or near the active center appear to protect the enzyme only if, by interacting strongly with the esteratic site, they physically obstruct its approach. Its reaction is not disturbed by non-specific adsorption of these molecules in regions adjoining the active center, and is comparatively insensitive to conditions that reversibly denature the enzyme. The binding of three classes of substrates is discussed: acetylcholine analogs, phenyl esters, and indophenyl acetate. Acetylcholine fits into an active center crevice and covers both the anionic and esteratic sites, but bulkier analogs, which cannot fully penetrate, are held primarily at the anionic site and adjoining non-polar regions, with weak and intermittent attraction to the esteratic site. Phenyl esters are bound within the crevice, over the esteratic site. Indophenyl acetate is adsorbed on the margin of the active center, covering neither the anionic nor esteratic site.

INTRODUCTION

Substrate binding and catalysis by enzymes are probably not separable processes, since the »freezing« of the substrate molecule in the active center is now regarded as a major contributor to catalytic efficiency[1]. In acetylcholinesterase (AChE) the active center consists of two adjoining parts, one, a binding (anionic) site, and another, a catalytic (esteratic) site, and it is therefore possible for substrate molecules to become bound at the former while interacting either weakly or strongly with the latter, with more or less predictable effects on catalytic rates. This subject is explored here, with the aid of methanesulfonyl fluoride (MSF)* as an active site probe. Observations are described on three classes of substrates: (1) analogs of acetylcholine (ACh); (2) phenyl esters; and (3) a substrate in a class by itself, indophenyl acetate.

MSF reacts with AChE to form a sulfonylated enzyme derivative, probably at the esteratic site[2-4]. For reasons discussed below a molecule bound in or near the active center will normally prevent reaction with MSF only if it obstructs access to the site of reaction. It does not do so if it simply reduces normal catalytic activity, as a result, for example, of non-specifically adsorbing to the enzyme and as in the case of denaturing agents perturbing the protein structure and disarranging the components of the esteratic site.

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* Abbreviations: AChE, acetylcholinesterase; MSF, methanesulfonyl fluoride; ACh, acetylcholines; DFP, diisopropylphosphorofluoridate.

Because of this, as well as on account of its small size, MSF is particularly suited for investigating the extent to which bound substrate molecules interact with the esteratic site before they react with it.

The first point to be made is that reaction with MSF is rather insensitive to many agents which cause unspecific perturbations of enzyme structure, as in reversible denaturation. Table I shows relative rates of reaction of enzyme with MSF, diisopropylphosphorofluoridate (DFP), and a neutral substrate, 4-nitrophenyl acetate, in the presence of a variety of denaturing agents. All 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_m is much lower than that of ACh)[5]. Reaction with MSF is seen to be not much slower under conditions that inhibit the other reactions, and in two cases is actually accelerated: with n-butanol, where the increase in rate is slight, and with guanidine-HCl, where it is large.

TABLE I.

Relative rates of reaction of eel acetylcholinesterase with 1.8×10^{-5} M diisopropyl-phosphorofluoride (DFP), 1.0×10^{-3} M p-nitrophenyl acetate (NPA) and 5.8×10^{-4} M methanesulfonyl fluoride (MSF) in the presence of various agents. Temp. 26 °C, pH = 8.0, 20 mM potassium phosphate buffer.

Agent	Concentration	Relative Rates of Reaction*		
		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	4%	0.0	0.12	0.7
Dimethylsulfoxide	3%	0.0	0.17	0.4
m-Dinitrobenzene	0.5 mM	0.2	0.25	0.9
Guanidine HCl	0.3 M	0.8	0.6	2.7

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

Other studies have shown that many cations can accelerate inactivation by MSF even though they are bound by non-specific forces in regions near but not directly over the anionic site (*i. e.* in a manner that would reduce acetylation rates, were they substrates). For example in the case of fly AChE, the large ions tetrapentyl ammonium and tetraphenyl arsonium accelerate the reaction, though were their centers of positive charge lined up at the same point as the quaternary nitrogen atom of ACh they should overlap the esteratic site[5]. Such ions protect against DFP[6] and inhibit substrate hydrolysis, indicating that they are probably bound near the site of reaction. Obviously the specific interactions which occur between ACh and the active center are not a requirement for cation-induced acceleration. The mechanism by which this occurs is still uncertain but could be due to the proximity of the bound cation and the leaving fluoride anion of MSF.

These findings cast doubt on interpretations of the phenomenon which assume specific interactions with bound ligands, and specific conformational

changes, such as should occur in an induced fit or allosteric mechanism. Operation of the latter is, in the case of substrates, a possibility, since the enzyme is a tetramer of $\alpha_2\beta_2$ structure[7] and since there is disagreement as to the number of catalytic sites, either two or four[8,9]. An allosteric mechanism for MSF activation, in which binding at one site of a multi-site enzyme induces changes in conformation at other sites, suffers from two further objections. First, the telegraphed effects from an allosteric site make the catalytic site more reactive with one relatively non-specific active-site reagent, MSF, but very much less reactive with another, DFP, as well as substrates, and this is unexpected. In addition, substrates at concentrations above their K_m values must become bound at the catalytic site and should then protect against MSF, but this does not happen[5]. Accordingly the observations on substrates described here are most reasonably explained in terms of various kinds of attachment in or near a single active center.

The first group of substrates to be discussed includes analogs of ACh in which the quaternary ammonium group is enlarged. These substrates are found to have a lower maximum velocity than ACh, and surprisingly they are more weakly bound, even though in the corresponding choline series the analogs with bulky non-polar substituents on the nitrogen atom are more firmly bound. An important correlation emerges when these observations are compared with effects on rates of enzyme inactivation by MSF. Choline and its analogs accelerate this reaction, but good substrates, such as ACh, protect the enzyme. However, with increasing bulk, substrates lose this property and as their affinities and maximum velocities decline protection gives place to acceleration. Some of this information is summarized in Table II. A particularly

TABLE II.

Affinities, substrate activities and effects on methanesulfonyl fluoride (MSF) reaction for acetylcholine and choline analogs ($R_3N^+C_2H_4OCOCH_3$ and $R_3N^+C_2H_4OH$), from ref. 4.

Enzyme	R_3	Ester			Alcohol		$\dfrac{\text{Affinity, ester}}{\text{Affinity, alcohol}}$
		Relative Affinity*	Relative Acetylation Rate	k/k_o (MSF)	Relative Affinity	k/k_o (MSF)	
Fly AChE	Me_3	1.0	1.0	0.14	1.0	—	7.3
	Me_2Bu	0.5	0.66	0.79	1.5	—	2.4
	Et_2Bu	0.2	0.30	7.5	4.0	—	0.35
Bovine AChE	Me_3	1.0	1.0	0.15	1.0	5.3	1.7
	Me_2Bu	1.0	<0.25	2.4	3.3	2.7	0.5
	Et_2Bu	0.7	<0.08	—	1.7	4.7	0.72

* With acetylcholine and bovine acetylcholinesterase, deacetylation is rate-limiting and therefore K_m overestimates the affinity. The affinity value used here was taken from the K_m for dimethylaminoethyl acetate (which is limited at acetylation) corrected for an additional methyl group, from the ratio of afinities of choline and dimethylaminoethanol. This should underestimate the binding contribution of the acetyl group, making the ratio of affinities of acetylcholine and choline too low. In the case of other substrates, and with acetylcholine and fly acetylcholinesterase, acetylation is rate-limiting, and K_m values were used as measures of affinity.

illuminating case is that of the N-butyl analog of ACh (N,N,N-dimethylbutyl aminoethyl acetate). This is a rather poor substrate of bovine erythrocyte AChE, the acetylation reaction being less than 1/4 that for ACh. It is less firmly bound than the alcohol from which it is derived, a somewhat surprising observation since the acyl group could be expected to contribute positively to binding, and in the case of good substrates does. It is found to accelerate inactivation by MSF. With fly AChE the same ester is a relatively good substrate, due to somewhat less stringent specificity requirements. Its acetylation rate on the enzyme is 2/3 that of ACh, and it is more firmly bound than the parent alcohol. Now, instead of accelerating MSF reaction, it protects.

These correlations are readily explained if all cationic substrates initially bind in the anionic site and if the ester group of the substrate then makes intermittent contact with the esteratic site, the frequency of contact depending on the detailed complementarity between the active center and the substrate. Infrequency of contact, *i. e.* weak interaction with the esteratic site, would necessarily be associated with a low catalytic rate, low substrate affinity, and weakened protection against MSF. Thus a good substrate such as ACh involves the entire active center in its attachment, while poor substrates involve only part.

It is necessary at this point to say something about the occurrence of the acetyl enzyme intermediate formed in the course of substrate hydrolysis. This intermediate could not react with MSF because substrates and MSF are believed to acylate the same chemical group in the enzyme[3,4]. Therefore, when deacetylation is the slow step in substrate hydrolysis and the acetyl enzyme is the predominant form once the substrate is bound, we expect substrate protection regardless of the nature of the interactions in the first enzyme-substrate complex. This is the case with bovine erythrocyte AChE and ACh, where deacetylation is probably rate-limiting. Fortunately for the present study, the insect enzyme is different, and acetylation appears to be rate--limiting with all substrates, including ACh[10]. Protection therefore means, in this case, that ACh strongly interacts with esteratic site. Of course with substrates whose V_m is lower than that of ACh there can be no doubt that acetylation is rate-limiting, and here the interpretation is straight-forward.

The conclusion from this part of the work is that the active center can be regarded as a crevice, into which bulky substrates cannot properly fit. The idea of a crevice appears to be required to explain why bulky N-alkyl analogs of ACh are more weakly bound than the corresponding choline analogs (Table II). Hence the affinity of substrates tends to diminish with added bulk. Obstruction at the anionic site also tips the molecule out of the active center, placing the ester group too far from the esteric site to obstruct the approach of MSF. A further consequence is that the substrate reacts slowly, and acetylation instead of deacetylation becomes rate-limiting. These ideas are illustrated in Figures 1a and 1b.

Our second class of substrates consists of phenyl esters. This class is easily dealt with, because protection appears to occur in all cases, including that of 3-mercaptophenyl trimethylammonium acetate. This is a cationc substrate, like those above, and it has a relatively low V_m[5]. As an ACh analog, therefore, it should accelerate inactivation. Instead it protects, and all this, together with the fact that phenyl acetate has an extremely high maximum velocity,
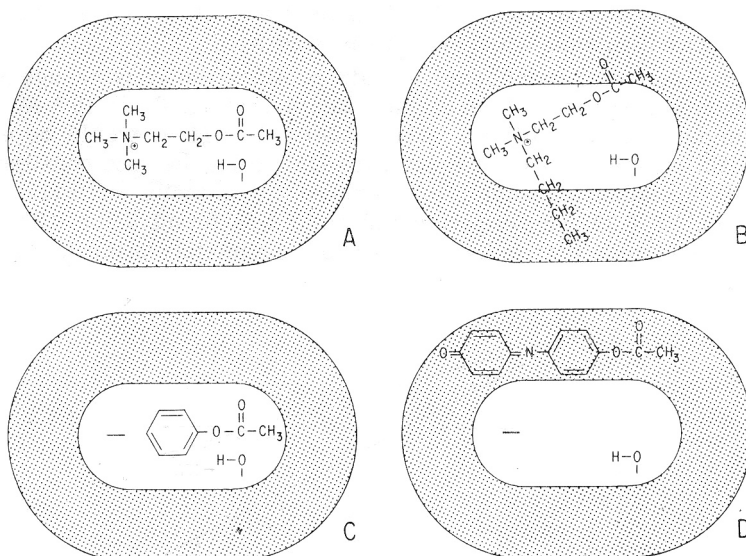
Fig. 1. Proposed interaction of four substrates with the active center of acetylcholinesterase. The inner light area represents the active center crevice; the anionic site is on the left and the esteratic site, which contains a serine hydroxyl group (OH), on the right. The surrounding shaded area represents a region of the protein that nonspecifically adsorbs non-polar molecules.

(A) Acetylcholine completely fills the active center.

(B) N,N,N-dimethyl-n-butylaminoethyl acetate is too large to fit into the crevice, and this prevents its acetyl group from interacting closely with the esteratic site. Hence it is slowly hydrolyzed and fails to protect against MSF.

(C) Phenyl acetate fits into the active center, is rapidly hydrolyzed and protects against MSF.

(D) Indophenyl acetate is bound on the margin of the active center. Consequently it is slowly hydrolyzed and it fails to protect against MSF.

suggests that a phenyl binding region is closely associated with the esteratic site and probably forms part of the crevice between the two sub-sites (Fig. 1c).

The last of our substrates is indophenyl acetate, which is of special interest because it exhibits the following unusual behavior. AChE treated with irreversible inhibitors that react in or near the anionic site (those of the aziridinium class[11-13] and p-(trimethylammonium) benzene diazonium fluorborate[14]) is more efficient in catalyzing hydrolysis of this substrate than is the native enzyme, but very much less efficient with all other substrates. Also enzyme treated with tetranitromethane, a reagent for tyrosine, loses all activity with acetyl-thiocholine, but is fully active with indophenyl acetate[15]. Indophenyl acetate is reported to inhibit ACh hydrolysis in a non-competitive manner, which is very odd if the same active center hydrolyzes both substrates, and its hydrolysis is non-competitively inhibited by tetraethylammonium and decamethonium[16].

Experiments were carried out on the effect of indophenyl acetate on rates of enzyme inacivation, first by MSF and then by DFP. Enzyme activity was simultaneously assayed from rates of hydrolysis of this substrate (making use of indophenol absorption at 625 nm) and of acetylthiocholine (small samples for which were withdrawn at intervals from the reaction mixture). The tracing of indophenyl acetate hydrolysis curved off with time, as the enzyme lost activity, and rates were determined from tangents on this tracing. It is

seen in Figs. 2 and 3 that under inhibition by either MSF or DFP, the rate of inactivation was identical, whichever assay was used.

It is also seen that indophenyl acetate protects the enzyme against DFP (Fig. 3). From the concentration of indophenyl acetate, 6.6×10^{-4} M, and its K_m value under these conditions, $(4.54 \pm 0.04) \times 10^{-4}$ M, it would be expected that if the ES complex does not react with DFP then the rate constant in the presence of the substrate should be reduced by a factor of $1/(1 + [S]/K_m)$, which is equal to 0.39. The experimental ratio was found to be 0.37. It must therefore be concluded that indophenyl acetate completely protects the enzyme against DFP.
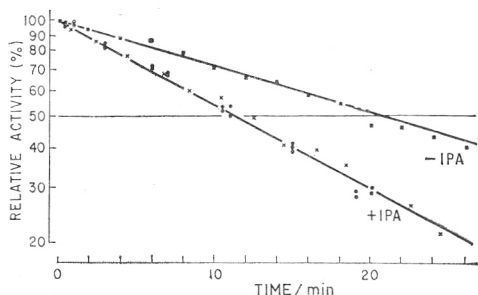

Fig. 2. Inactivation of eel AChE by 1.45×10^{-4} M methanesulfonyl fluoride, either in the presence or absence of 6.6×10^{-4} M indophenyl acetate (IPA). Enzyme activity was determined with acetylthiocholine in both cases. In the presence of IPA, activity in the acetylthiocholine assay is denoted by a circle, and that in the IPA assay by a cross. The calculated second order rate constants are 2.3×10^2 M^{-1} min^{-1} in the absence of IPA and 4.2×10^2 M^{-1} min^{-1} in its presence. Temp. 26 ^{0}C, pH = 8.0, in 20 mM potassium phosphate buffer.
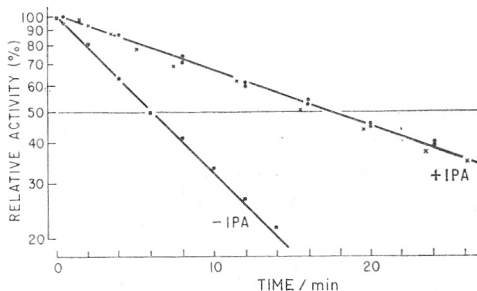

Fig. 3. Inactivation of eel AChE by 5.8×10^{-6} M diisopropylphosphorofluoridate in the presence or absence of 6.6×10^{-4} M IPA. Conditions and assays as in Fig. 1. The calculated second order rate constants are 1.9×10^4 M^{-1} min^{-1} in the absence of IPA and 7.0×10^3 M^{-1} min^{-1} in its presence.

These two observations, identical inactivation rates in either assay, and complete protection by indophenyl acetate against DFP, argue that the same active center acts upon both substrates. A further important observation is that the rate of inactivation by MSF is almost twice as high in the presence of indophenyl acetate as in its absence. Like bulky ACh analogs, therefore, indophenyl acetate may become bound in the region of the active center but may interact weakly with the esteratic site. In this case the reason for activation (of MSF reaction) is not at all clear, though as seen in Table I a small activation occurs in the presence of n-butanol and a large one in the presence of guanidine-HCl.

In interpreting the findings on indophenyl acetate it should be noted that this substrate is more unstable in imidazole-catalyzed hydrolysis than is phenyl acetate, and yet with the enzyme it has a V_m only 2% as high. If bound in the same position its intrinsic chemical properties would lead one to expect rapid hydrolysis. This suggests that it must be bound elsewhere, and the experiments described above on bulky ACh analogs, which led to the concept of an active site crevice only big enough for ACh[5], would lead one to expect that indophenyl acetate, a considerably larger molecule should fail to enter the crevice. The experiments with anionic site-directed inhibitors and with MSF support this, since they indicate that bound indophenyl acetate interacts strongly with neither the anionic nor esteratic site. It does interact with the latter, but only intermittently, just as do certain analogs of ACh. Thus the substrate appears to lie alongside, but not within the active site crevice (Fig. 1d). Chemical modifications in the region of the anionic site alter its orientation, increasing the probability of contact between its acetyl group and the esteratic site, and so accelerating hydrolysis. Such modification would necessarily disorient a specifically bound substrate molecule, such as ACh, blocking its reaction, but with a substrate that is normally adsorbed in an unfavorable orientation, the possibility must exist that the orientation will be improved.

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DISCUSSION

U. Brodbeck:

You were mentioning briefly the effects of proflavine on AChE. Would you please elaborate on this point.

R. M. Krupka:

Unfortunately, I can add little to what I have said. Proflavine reversibly inhibits AChE, and at these concentrations, where we know it binds, it accelerates the rate of inactivation by methanesulphonyl fluoride.

R. D. O'Brien:

With respect to special properties of indophenyl acetate, I do not see that your experimental findings differ from those we reported — in particular we also showed that the rate of enzyme phosphorylation was identical as measured by indophenyl acetate or other substrates implying that all use the same catalytic OH. As for

the interpretation, the only way in which yours differs from ours is that you arbitrarily specify that the indophenyl acetate binding site (clearly different from the acetylcholine binding site) is identical with a »rim around the active site« rather than assigning indophenyl acetate its own site. There appears to be no experimental evidence for your specification.

R. M. Krupka:

The important point here is that your work with indophenyl acetate was done with erythrocyte AChE, while the experiments I showed were on the eel enzyme. I have also looked at erythrocyte AChE (the purified preparation from Sigma) and the results were not so simple as with eel enzyme. The loss of activity during methane sulphonyl fluoride or DFP inhibition was definitely higher when assayed with acetylthiocholine than with indophenyl acetate. The difference was not large and we in fact missed it in our initial experiments. The explanation is probably that there is a small impurity in the preparation that acts upon indophenyl acetate. but not acetylthiocholine. This impurity would not alter the behaviour of the preparation with normal substrates, but it complicates things here. An impurity now seems a safe interpretation, but only because we know that with eel enzyme the rates are identical. As to the involvement of a single active centre, a second step in the argument is the present demonstration that the degree of protection against DFP by indophenylacetate at a non-saturating concentration is almost exactly what is predicted from the K_m value. The new methanesulphonyl fluoride results provide unequivocal evidence that interaction of indophenyl acetate with the esteratic site must be very weak.

W. N. Aldridge:

Do the acetylcholine analogues produce substrate inhibition?

R. M. Krupka:

Acetylcholine analogs with a relatively low maximum velocity did not show substrate inhibition at concentrations similar to those for acetylcholine. Some inhibition was observed in one or two cases, as I recall, but it only occurred at much higher substrate concentrations, which is what was expected if inhibition of deacetylation is involved.

I. Silman:

Do dimethylformamide and dimethylsulfoxide cause total inhibition of DFP inactivation of AChE, and is this irreversible?

R. M. Krupka:

Inhibition of DFP reaction is practically complete. We did not determine whether enzyme preincubated with dimethylformamide or dimethylsulfoxide was irreversibly changed in its reaction with DFP, though I should point out that after dilution, the enzyme had unchanged activity with acetylthiocholine.

E. A. Barnard:

Am I correct in assuming that no acceleration by cationic ligands of the inhibition reaction has ever been shown with an organophosphate inhibitor, in contrast to the cases of methyne sulfonyl fluoride and carbamates?

R. M. Krupka:

Yes.

E. A. Barnard:

This being so, how is it to be interpreted, since in reactions with a compound such as DFP, cationic ligands in part retard rather than accelerate, and there is *a priori* reversible binding (as Main showed) of the inhibitor. Possibly it could occlude the anionic site in the active center with a more peripheral interaction. Is the distinction between the two types of inhibitor just due to a size difference? How does this fit in with your model of the active center?

R. M. Krupka:

The only definite reason I can think of that could explain the distinction, is the small size of methanesulphonyl fluoride compared to organophosphorus inhibitors, even the smallest, such as dimethylphosphorofluoridate, whose reaction with the enzyme is blocked by tetramethylammonium and tetraethylammonium ions. Consequently, there may be steric interference at the anionic site with organophosphorus inhibitors, but not with methanesulphonyl fluoride. As pure speculation one might assume that due to the dimensions of the »crevice« or to the chemical nature of the acylating agent the orientation of the bond to fluorine in the esteratic site is different for the two kinds of inhibitor, activation being assumed to depend on an alignment of the fluorine atom and the adsorbed cation.

SAŽETAK

Metansulfonil-fluorid: Istraživanje interakcije supstrata s katalitičkim mjestom acetilkolinesteraze

R. M. Krupka

Metansulfonil-fluorid (MSF), koji inhibira aktivnost acetilkolinesteraze vezivanjem za katalitičko mjesto, korisno je sredstvo za istraživanje vezivanja supstrata, i to stoga što molekule, adsorbirane blizu ili u samom aktivnom centru, mogu zaštititi enzim samo ako se čvrsto vežu za estersko mjesto te time fizički onemoguće pristup ovom spoju. Nespecifična adsorpcija molekula u blizini aktivnog centra ne ometa reakciju s MSF a do stanovite mjere ta je reakcija neosjetljiva i na uvjete pod kojima se enzim denaturira. Istražen je način interakcije triju vrsta supstrata: analoga acetilkolina, fenil esterâ i indofenil-acetata. Acetilkolin pristaje uz aktivni centar i pokriva obje, anionsku i estersku stranu. Prostorno veći analozi tek djelomično ulaze u aktivni centar, i to u anionsku stranu i u nepolarna područja u njenoj blizini, a samo djelomično ispoljavaju slabu privlačnost spram esterske strane. Fenil esteri vezani su na esterskoj strani unutar aktivnog centra. Indofenil-acetat adsorbira se uz granični dio aktivnog centra, a ne prekriva ni anionsku ni estersku stranu.

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