Hydrolysis of Electronically and Sterically Defined Substrates of Acetylcholinesterase*

Gilbert R. Hillman† and Henry G. Mautner‡

ABSTRACT: Sulfur- and selenium-containing analogs of acetylcholine were studied as substrates of electric eel acetylcholinesterase (EC 3.1.1.7). When sulfur or selenium was substituted for the acyloxy oxygen atom of acetylcholine, the binding to the enzyme was increased, but the rate of formation of the acyl enzyme intermediate was reduced. Inhibition of the hydrolysis of these substrates by benzoylcholine was studied; benzoylcholine was a noncompetitive inhibitor of the hydrolysis of acetylcholine, and to a much lesser extent of acetylthiolcholine, but a competitive inhibitor of the hydrolysis of acetylselenolcholine. This finding supports the suggestion that acylation of the enzyme becomes slower for acetylthiolcholine than for acetylcholine and becomes rate determining for acetylselenolcholine.

Acetylcholinesterase (AcChE),[1] the enzyme responsible for the rapid hydrolysis of the vital neurotransmitter acetylcholine (AcCh), has been the subject of numerous investigations. In particular, the mechanisms of this enzyme's interactions with substrates and with inhibitors have been explored to obtain information about the active sites involved. Some representative references include: Nachmansohn and Wilson (1951), Wilson and Cabib (1956), Belleau and Lacasse (1964), Krupka and Laidler (1961), Krupka (1967), and Bracha and O'Brien (1968). These studies lead to the concept that the catalytic functions of AcChE involve an "anionic site" to which the trimethylammonium group of AcCh is attracted, an "esteratic site" carrying the acyl group before the deacetylation step (which is rate limiting in the hydrolysis of AcCh), and "hydrophobic patches."

When the interactions of macromolecules and small molecules are studied it is customary to ignore the fact that even very minor modifications of a substrate or an inhibitor may alter conformation and electron distribution throughout these molecules. Thus, when modifying a substrate or inhibitor alters its ability to interact with an enzyme, it is difficult to ascertain whether steric or electronic factors are responsible for the observed changes in the interaction. To minimize these factors, we have been studying the interaction of AcChE with sterically and electronically defined analogs of AcCh and of related compounds.

The present work is concerned with the hydrolysis by AcChE of AcCh, acetylthiolcholine, and acetylselenolcholine. When the acyloxy oxygen atom of AcCh is replaced by sulfur, the conformation of the molecule is altered drastically. While the N^+CCO grouping of AcCh assumes the *gauche* (sc) conformation, in which the trimethylammonium group is fairly close to the acyloxy oxygen, either in the crystal (Canepa *et al.*, 1966) or in solution (Culvenor and Ham, 1966), the corresponding N^+CCS grouping in AcSCh is in the fully extended *trans* (ap) conformation in either medium (Shefter and Mautner, 1969; Cushley and Mautner, 1969). However, it proved possible to demonstrate that AcSCh and AcSeCh are essentially isosteric either in the crystal or in D_2O solution (Shefter and Mautner, 1969). Therefore, differences in the abilities of the latter substrates to be hydrolyzed by AcChE are likely to be due to electronic rather than to steric factors. Since kinetic methods make it possible to investigate some details of the acylation and deacylation steps involved in substrate–AcChE interactions, a detailed investigation of the enzymic hydrolysis of the above compounds seemed of interest.

Materials and Methods

2-Dimethylaminoethyl acetate, the corresponding thiol and selenol esters, and AcSeCh, were synthesized in this laboratory (Günther and Mautner, 1964).

Electric eel AcChE was a gift from the laboratory of Dr. David Nachmansohn of Columbia University. The preparation used in most of these studies had a specific activity of 130 mmol of AcCh hydrolyzed per hr per mg of protein.

Enzyme assays were carried out by titration of product with 0.01 N NaOH, at pH 7.5 ± 0.02 and 25° using a Radiometer autotitrator.

The observed initial rates (constant for at least 2 min) and substrate concentrations were used to construct weighted double-reciprocal plots (Wilkinson, 1961). The K_m and V_{max} values were determined as $-1/x$ intercept and $1/y$ intercept, respectively. The variance of the K_m was estimated from the dispersion of the data points around the computed line, using the method of Wilkinson (1961) and equations derived

* From the Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510. *Received January 12, 1970.* This is paper XI in the series: Sulfur and Slenium Compounds Related to Acetylcholine and Choline. The previous paper in the series is by Chu and Mautner (1970). This work was supported, in part, by grants from the National Science Foundation (GB-6835) and the National Institute of Neurological Diseases (NS-07853).

† In partial fulfillment of the requirements for the degree of Doctor of Philosophy (U. S. Public Health Service Trainee, Grant GM-0059).

‡ Present address: Department of Biochemistry and Pharmacology, Tufts University School of Medicine, Boston, Mass. 02111.

[1] Abbreviations used are: AcCh, acetylcholine; AcChE, acetylcholinesterase, AcSCh, acetylthiolcholine, AcSeCh, acetylselenolcholine.

by Dr. Y.-K. Chan of the Yale School of Epidemiology and Public Health.

In terms of the following reaction scheme: (Wilson and Cabib, 1956; Krupka and Laidler, 1961)

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E + P$$

where ES is the enzyme–substrate complex, EA is the acyl enzyme. The experimental quantities K_m and V_{max} have the following significance (Wilson and Alexander, 1962):

$$V_{max} = \frac{k_2[E]_0}{1 + \frac{k_2}{k_3}} \quad (1)$$

where $[E]_0$ = total enzyme

$$K_m = \frac{k_{-1} + k_2}{k_1\left(1 + \frac{k_2}{k_3}\right)} \quad (2)$$

If K_s is defined as k_{-1}/k_1, the dissociation constant of the substrate and the enzyme, then from eq 2, assuming k_2 is much slower than k_{-1}, one can obtain:

$$K_s = K_m\left(1 + \frac{k_2}{k_3}\right) \quad (3)$$

The value of k_2/k_3 for a substrate may be determined using eq 4 which is based on eq 1. The derivation of this equation and the assumptions made are summarized in the Appendix.

$$\frac{k_2}{k_3} = \frac{1}{1.2\left(\frac{V_{max_a}}{V_{max_s}}\right) - 1} \quad (4)$$

Having determined k_2/k_3 using this equation, one can determine K_s using eq 3.

For an inhibitor which blocks deacylation by combining with the acyl enzyme with a dissociation constant, K_i' (Krupka, 1964), the observed inhibition constant, $K_i'_{obsd}$, is dependent on the relative rates of acylation and deacylation of the enzyme, k_2 and k_3.

The relation between the $K_i'_{obsd}$ and the K_i' is as follows (Krupka, 1964):

$$K_i'_{obsd} = K_i'\left(1 + \frac{k_3}{k_2}\right) \quad (5)$$

If k_3 is small compared with k_2, $k_i'_{obsd} \cong K_i'$. But if the opposite is true, then $K_i'_{obsd}$ becomes relatively large; that is, the observed noncompetitive component of inhibition disappears. If the relative values of k_2 and k_3 for a series of substrates are determined by use of eq 4, then the fact that an inhibitor combines with the acyl enzyme should result in those substrates for which acylation is slower than deacylation, showing less noncompetitive inhibition. The assumption that binding to the acyl enzyme leads to noncompetitive inhibition is compatible with the results obtained.

Results

The observed values of K_m and V_{max} for the quaternary and tertiary substrates are given in Table I. It is clear that substitution of sulfur or selenium for oxygen in the acyloxy position of the esters decreases the V_{max} and decreases the K_m. Table I also shows the values of k_2/k_3 and K_s calculated from eq 2 and 3, for all the substrates. The k_2/k_3 ratio decreases as substitutions are made in the acyloxy position. Since k_3 is assumed to be the same for all these substrates, this finding implies that the rate of acylation of the enzyme decreases when sulfur or selenium replaces the acyloxy oxygen.

TABLE I: Hydrolysis Constants of Acetylcholinesterase Analogs, $R(CH_3)_2N^+CH_2CH_2BC(O)CH_3$.

B	R	K_m ($\times 10^4$ M)	V_{max} (Arbitrary Units)	k_2/k_3	K_s ($\times 10^4$ M)
O	H	8.2 ± 2[a]	16[a]	0.8	14
S	H	3.4 ± 0.1[a]	15[a]	0.7	5.7
Se	H	0.55 ± 0.05[a]	4.2[a]	0.1	0.62
O	CH_3	0.95 ± 0.12	30 ± 1.3	5.7[b]	6.2
S	CH_3	0.56 ± 0.099	28 ± 0.2	1.8	1.4
Se	CH_3	0.14 ± 0.02	16 + 0.3	0.9	0.36

[a] Data obtained by T.-C. Chou (personal communication). [b] Determined by Wilson and Cabib (1956).

The values of K_s shown in Table I reveal that substitutions in the acyloxy position give a marked increase in the strength of binding to the enzyme; this increase would have been underestimated if the K_m had been interpreted as a dissociation constant, as is often done.

The inhibition by 1 mM benzoylcholine of the hydrolysis of several substrates of AcChE is shown in Figure 1A–D, in the form of double-reciprocal plots. The lines shown are fitted to the points by the weighted regression procedure of Wilkinson (1961). While benzoylcholine is a noncompetitive inhibitor of the hydrolysis of AcCh (Figure 1A), it is almost purely a competitive inhibitor of the hydrolysis of 2-dimethylaminoethyl acetate (Figure 1B). AcSCh shows a small but significant noncompetitive component to its inhibition (Figure 1C), as calculated at the 95% confidence level, a fact which is not apparent on inspection of the graph. The inhibition of the hydrolysis of AcSeCh is competitive (Figure 1D), within experimental error; there is no significant difference between the y intercepts. Under these experimental conditions benzoylcholine is hydrolyzed to a negligible extent.

Discussion

The assumption that the noncompetitive inhibition by benzoylcholine is due to the binding of the inhibitor to the acyl enzyme is consistent with the values of k_2/k_3 shown in Table I and with the data shown in Figure 1A–D. One would expect that if the inhibitor combines with the acyl enzyme, a noncompetitive component of inhibition would

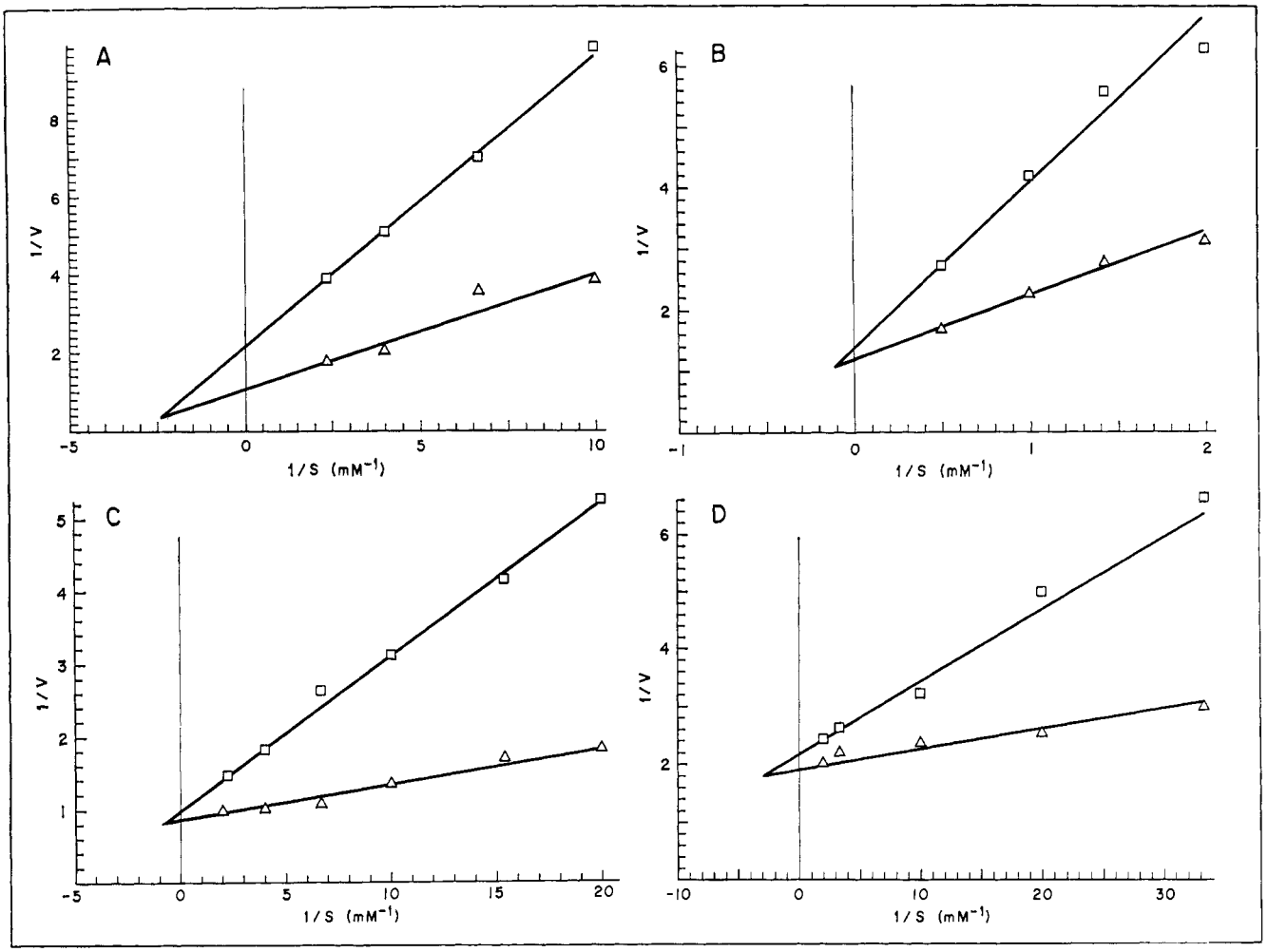

FIGURE 1: Hydrolysis of AcCh by AcChE: (A) inhibition by benzoylcholine (△) no inhibitor (□), [I] = 1 mM. Lines are calculated weighted regression lines. (B) Hydrolysis of 2-dimethylaminoethyl acetate by AcChE, inhibition by benzoylcholine. Symbols and lines are as in A. (C) Hydrolysis of AcSCh by AcChE, inhibition by benzoylcholine. Symbols and lines are as in A. (D) Hydrolysis of AcSeCh by AcChE, inhibition by benzoylcholine. Symbols and lines are as in A.

be conspicuous only if deacetylation of the enzyme were strongly rate limiting, as in the case of AcCh (Wilson and Cabib, 1956). In the case of substrates such as 2-dimethylaminoethyl acetate, for which deacetylation is not rate limiting (Wilson and Cabib, 1956), benzoylcholine becomes a competitive inhibitor (Figure 1B), rather than a noncompetitive inhibitor as in the case of AcCh (Figure 1A). These findings suggest that benzoylcholine combines with the acyl enzyme.

Assuming that benzoylcholine interacts with the acyl enzyme, we can then determine whether acylation (k_2) or deacylation (k_3) is rate limiting in the case of the thiol esters and selenol esters. One would expect that those substrates which we have shown to acylate the enzyme more slowly than does AcCh (all of which form the same acetyl enzyme) would also show less noncompetitive inhibition by benzoylcholine. The double-reciprocal plots in Figure 1A–D demonstrate that this is the case.

Theoretically, it would be possible to calculate the K_i' from the $K_i'_{obsd}$ values and the values of k_2/k_3 in Table I using eq 5. However, statistics show that such a calculation is not justified by the data.

The results show that substitution of the acyloxy oxygens of the substrates by sulfur or selenium affects the binding of these compounds to and the rate of their breakdown by AcChE. While in the hydrolysis of AcCh deacylation is rate limiting, acylation is rate limiting in the enzymic hydrolysis of AcSeCh, with the k_2/k_3 ratio being intermediate for the hydrolysis of AcSCh. Acylation is slower than deacylation for all the 2-dimethylaminoethyl compounds studied.

All of the compounds investigated form the identical acyl enzyme. The pK_a's of the dimethylamino groups of 2-dimethylaminoethyl esters, thiol esters, and selenol esters are very similar (Chu and Mautner, 1966), while AcCh, AcSCh, and AcSeCh have identical trimethylammonium groups. Furthermore, while the conformations of AcCh and AcSCh differ, the shapes and conformations of AcSCh and AcSeCh are known to be virtually identical even in solution. From these factors it can be deduced that the observed differences in the enzymic hydrolysis of the isologous esters, thiol esters, and selenol esters are not likely to be due to differences in the rate of breakdown of the acetyl enzyme (k_3), nor are they likely to be due to differences in the presumably very fast rate of formation of the enzyme–substrate complex

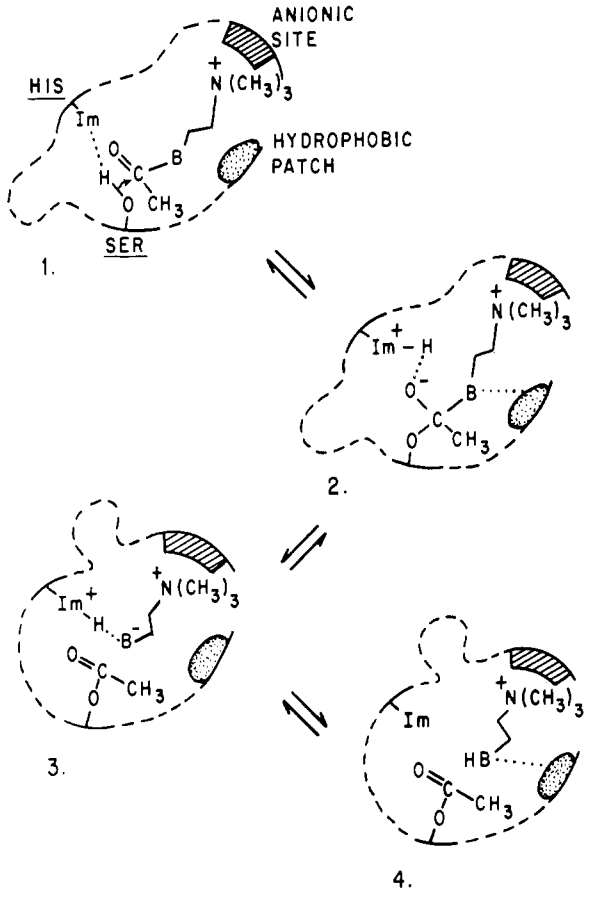

FIGURE 2: Suggested mechanism of acylation.

(k_1). If fit and flexibility of substrate to enzyme were rate determining in k_1, these factors would be extremely similar for the isosteric molecules AcSCh and AcSeCh.

Evidence has been accumulating that the interaction of hydrolytic enzymes with their substrates is too complex to be expressed purely in terms of acylation and deacylation. It has been suggested by Krupka that during the hydrolytic action of AcChE, intermediates are formed during the acetylation step, the formation and breakdown of which involve different active sites of the enzyme (Krupka, 1966, 1967). More recently, Caplow emphasized the importance of the formation and breakdown of the tetrahedral intermediate preceding the formation of the acyl enzyme during chymotrypsin catalysis (Caplow, 1969).

Krupka suggested the following sequence of events for the hydrolysis of AcCh by AcChE. After formation of the enzyme–substrate complex, presumably involving attraction of the trimethylammonium group of AcCh to the anionic site, a catalytic group catalyzes transfer of the acetyl group to a serine residue in the esteratic site while choline is lost. This process is believed to involve a conformational change bringing the acetyl residue into the vicinity of a group which catalyzes deacetylation.

Following this scheme, conversion of the enzyme–substrate complex into acyl enzyme involves several factors such as: a conformational change, which the trimethylammonium group presumably facilitates (Wilson and Cabib, 1956); formation of a tetrahedral intermediate by the substrate; and breakdown of the tetrahedral intermediate with the formation of acyl enzyme and the loss of alcohol.

It should be noted that the relative rates for the enzymic hydrolysis of AcCh analogs (AcCh > AcSCh > AcSeCh) are different from the relative rates of the nonenzymic hydrolysis of these compounds at a pH of 7.5. In the absence of enzyme, the relative rates for hydrolysis are AcSeCh faster than AcCh faster then AcSCh (D. Chou and H. G. Mautner, unpublished data). Furthermore, due to neighboring group catalysis, in the absence of enzyme, 2-dimethylaminoethyl, -thiol, and -selenol esters are hydrolyzed more rapidly at this pH than are their quaternary analogs (Chu and Mautner, 1966), while in the presence of enzyme the opposite is true.

If one assumes that AcSCh and AcSeCh are isosteric in the milieu of the active sites of AcChE as well as in water and if one assumes that these isologs possess similar rotational barriers, the abilities of these molecules to fit the active sites of AcChE should be very similar. Therefore, the observed differences in the enzymic hydrolysis of AcSCh and AcSeCh are uniquely free of steric complications.

It may be seen in Table I that the k_2/k_3 ratio is much greater for AcCh than for the other two substrates. This fact might be related to the difference in steric configuration between the thiol ester and the ester. The *gauche* configuration of the $OCCN^+$ group of AcCh may be particularly advantageous for the acylation of the enzyme, while the *trans* configuration of the other two substrates may be less advantageous.

It has been widely assumed that histidine is involved in the functions of the active site of AcChE (Bergmann *et al.*, 1950; Krupka and Laidler, 1961; Krupka, 1967) although the evidence is not as conclusive as it is in the case of the active site of chymotrypsin (Mathews *et al.*, 1967). There is no question about the importance of serine in the functions of AcChE (for a review, see Wassermann, 1968). Evidence has been presented (Wilson and Cabib, 1956; Kitz and Kremzner, 1968) that attachment of quaternary substrates to AcChE induces conformational changes the purpose of which may be the bringing into close proximity of serine, histidine, and the ester group to be hydrolyzed. The possibility of further changes in the conformation of enzyme, substrate, or both during the formation and breakdown of the tetrahedral intermediate cannot be excluded. A possible mechanism for the interaction of enzyme and substrate is depicted in Figure 2.

This mechanism differs from that postulated previously (Krupka, 1967) in that it involves only one instead of two imidazoles and assumes a rearrangement within the tetrahedral complex similar to that postulated for anilide hydrolysis by chymotrypsin (Caplow, 1969). The postulate of the catalytic functions of two imidazoles (Krupka, 1967) is based on the observation that during the acetylation reaction of AcChE a group with a pK_a of 5.3 functions in a slow step and a group with a pK_a of 6.3 functions in a fast step. However, it seems possible that the micro pK_a's of the imidazole nitrogens might be affected by a conformational change altering the dielectric constant of their environment. The rearrangement in the positioning of substrate, serine, and imidazole postulated as taking place in the tetrahedral complex might involve a conformational change in either enzyme or substrate.

On the basis of dipole moment and spectroscopic measurements, it is known that in passing from esters to thiol esters to selenol esters, carbonyl basicity, and the ability of carbonyl oxygen atoms to accept hydrogen bonds are progressively reduced, affecting formation of the tetrahedral intermediate

(Wallmark *et al.*, 1969, 1970). Similarly, the ability of the acyloxy oxygen atoms of esters to form hydrogen bonds presumably exceeds that of the sulfur and selenium atoms in analogous thiol esters and selenol esters affecting breakdown of the tetrahedral intermediate. Because of these considerations both the formation and the breakdown, according to the above mechanism, of the tetrahedral complex should proceed progressively more slowly in passing from AcCh to AcSCh to AcSeCh.

The relatively high lipid solubility of sulfur and selenium compounds as compared with that of their oxygen isologs (Mautner Clayton and, 1959; G. R. Hillman and H. G. Mautner, unpublished data) also raises the possibility that interaction of the sulfur and selenium atoms of AcSCh and AcSeCh with hydrophobic patches (Bracha and O'Brien, 1968; Aharoni and O'Brien, 1968; Belleau and Lacasse, 1964) near the esteratic site may interfere with the flexibility within the ES complex required for a conformational change of the enzyme-bound compound or of the enzyme itself during the acetylation reaction. Such interactions may also interfere with the release of cholinethiol or cholineselenol from the tetrahedral intermediates formed by AcSCh and AcSeCh. Nonproductive hydrophobic interactions might also decrease the ability of the substrates to interact with the reactive groups involved in acyl enzyme formation.

Finally, it should be noted that after formation of acetyl-AcChE, the choline, cholinethiol, or cholineselenol generated might still be transiently attached to the anionic site of the enzyme and thus exert an effect on deacetylation. It is well documented that trialkylammonium compounds can block deacetylation (Krupka, 1964, 1965). Thus, the assumption that k_3 must be identical for all the isologs of AcCh and of 2-dimethylaminoethyl acetate may be subject to question even though all these substrates form the identical acetyl enzyme.

Appendix

Equation 4 is derived as follows.

Let V_{max_a} be the V_{max} of AcCh, and V_{max_s} be the V_{max} of another substrate. Then, from eq 1,

$$\frac{V_{max_s}}{V_{max_a}} = \frac{\dfrac{k_{2_s}[E]_{0_s}}{1 + \dfrac{k_{2_s}}{k_{3_s}}}}{\dfrac{k_{2_a}[E]_{0_a}}{1 + \dfrac{k_{2_a}}{k_{3_a}}}} \tag{6}$$

where $[E]_0$ is the total enzyme concentration and k_2 and k_3 are the rate constants of acylation and deacylation of the enzyme. The subscripts a and s refer, respectively, to AcCh and to the other substrate.

If we use the same amount of enzyme in all experiments, we can say $[E]_{0_s} = [E]_{0_a}$, which we may call $[E]_0$. Also, if the substrate other than AcCh is an acetate ester we may assume that it forms the same acetylated enzyme as that formed by AcCh, and therefore we may assume that $k_{3_s} = k_{3_a}$, which we may then call k_3. We may then divide top and bottom of the fraction in eq 6 by $k_3[E]_0$, giving

$$\frac{V_{max_s}}{V_{max_a}} = \frac{\dfrac{k_{2_s}}{k_3 + k_{2_s}}}{\dfrac{k_{2_a}}{k_3 + k_{2_a}}} \tag{7}$$

Since it is known (Wilson and Cabib, 1956) that $k_{2_a}/k_3 = 5.7/1$; the bottom half of this fraction can be evaluated as $5.7/6.7 \sim 0.85$. We then obtain:

$$0.85 \times \frac{V_{max_s}}{V_{max_a}} = \frac{k_{2_s}}{k_3 + k_{2_s}} \tag{8}$$

If both sides of this equation are inverted, we obtain:

$$1.2 \times \frac{V_{max_a}}{V_{max_s}} = \frac{k_3 + k_{2_s}}{k_{2_s}} = 1 + \frac{k_3}{k_{2_s}} \tag{9}$$

Subtracting 1 from both sides and inverting, we finally have:

$$\frac{k_{2_s}}{k_3} = \frac{1}{1.2\dfrac{V_{max_a}}{V_{max_s}} - 1} \tag{10}$$

References

Aharoni, A. R., and O'Brien, R. D. (1968), *Biochemistry 7*, 1538.

Belleau, B., and Lacasse, J. (1964), *J. Med. Chem. 7*, 768.

Bergmann, F., Wilson, I., and Nachmansohn, D. (1950), *J. Biol. Chem. 186*, 693.

Bracha, P., and O'Brien, R. D. (1968), *Biochemistry 7*, 1545, 1555.

Canepa, F. P., Pauling, P., and Sörum, H. (1966), *Nature 210*, 907.

Caplow, M. (1969), *J. Amer. Chem. Soc. 91*, 3639.

Culvenor, C. C. J., and Ham, N. S. (1966), *Chem. Commun.*, 537.

Cushley, R. J., and Mautner, H. G. (1969), Abstracts of the 3rd International Biophysics Congress, Cambridge, Mass., Sept 1969, IO-10.

Cushley, R. J., and Mautner, H. G. (1970), *Tetrahedron* (in press).

Chu, S. H., and Mautner, H. G. (1966), *J. Org. Chem. 31*, 308.

Chu, S. H., and Mautner, H. G. (1970), *J. Med. Chem. 13*, 214.

Günther, W. H., and Mautner, H. G. (1964), *J. Med. Chem. 7*, 229.

Kitz, R. J., and Kremzner, L. T. (1968), *Mol. Pharmacol. 4*, 104.

Krupka, R. M. (1964), *Biochemistry 3*, 1749.

Krupka, R. M. (1965), *Biochemistry 4*, 429.

Krupka, R. M. (1966), *Biochemistry 5*, 1983.

Krupka, R. M. (1967), *Biochemistry 6*, 1183.

Krupka, R. M., and Laidler, K. J. (1961), *J. Amer. Chem. Soc. 83*, 1445.

Mathews, B. W., Sigler, P. B., Henderson, R., and Blow, D. M. (1967), *Nature 214*, 652.

Mautner, H. G., and Clayton, E. M. (1959), *J. Amer. Chem. Soc. 81*, 6270.
Nachmansohn, D., and Wilson, I. (1951), *Advan. Enzymol. 12*, 259.
Shefter, E., and Mautner, H. G. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 1253.
Wallmark, I., Krackov, M. H., and Mautner, H. G. (1969), 158th National Meeting of the American Chemical Society, New York, N. Y., Sept 1969, ORGN 156; *J. Amer. Chem. Soc.* (in press).
Wallmark, I., Krackov, M., and Mautner, H. G. (1970), *J. Amer. Chem. Soc.* (in press).
Was ermann, O. (1968), *Pharmazie 23*, 49.
Wilkinson, G. N. (1961), *Biochem. J. 80*, 324.
Wilson, I., and Alexander, J. (1962), *J. Biol. Chem. 237*, 1323.
Wilson, I., and Cabib, E. (1956), *J. Amer. Chem. Soc. 78*, 202.

Kinetics of Complex Formation between Human Carbonic Anhydrases and Aromatic Sulfonamides*

P. W. Taylor, R. W. King, and A. S. V. Burgen

ABSTRACT: The kinetics of the reaction between human carbonic anhydrases B and C and aromatic sulfonamides have been followed using the stop-flow method by measuring changes in protein fluorescence associated with complex formation. Affinity constants calculated from the ratio of kinetic constants are in agreement with those measured by equilibrium titration for sulfonamides which quench the tryptophan fluorescence. By using a coupled reaction system it is possible to measure dissociation rates for both quenching and nonquenching sulfonamides. With corresponding equilibrium affinities, association rates for nonquenching inhibitors can also be evaluated. The two enzymes show marked differences in specificity for the series of sulfonamides. Thermodynamic parameters have been determined and the complex is stabilized primarily through a favorable enthalpy change. Rate and thermodynamic data both indicate that the association rate is not diffusion controlled but that the formation of the complex requires a distinct activation energy. In contrast to most ligand–metal substitution processes, differences in the thermodynamic stability of the sulfonamide complexes are largely a consequence of the variation in the association rate constants.

The aromatic and heterocyclic sulfonamides form a group of highly active inhibitors possessing unusual selectivity for the metalloenzyme, carbonic anhydrase (hereafter referred to as enzyme) (Mann and Keilin, 1940; Maren, 1967). Although an unsubstituted SO_2NH_2 group attached to a conjugated ring system is required for inhibition, substantial modifications of ring structure are possible with the retention of the characteristic high affinity. Sulfonamides thus present for interaction a relatively simple and rigid molecular structure to which a large number of ring substitutions can be made. The crystal structure of the human enzyme C–acetoxymercurisulfanilamide complex at 5.5 Å shows that this inhibitor binds within a cleft on the enzyme surface with the sulfonamido group closely positioned with respect to the coordination sphere of the Zn (Fridborg *et al.*, 1967). Replacement of Zn(II) by Co(II) gives visible Co spectra characteristic of ligand geometry and symmetry around the transition metal (Lindskog, 1963). Formation of the sulfonamide complex is accompanied by changes in the energy of the d–d transitions of the Co enzyme (Lindskog, 1963). This evidence supports the concept that the sulfonamide–carbonic anhydrase complex is coordinated through a ligand–metal bond. In addition, an enhancement of quantum yield obtained with the fluorescent probe, 5-dimethylaminonaphthalene-1-sulfonamide, upon binding indicates that the aromatic ring system of this inhibitor is associated with a hydrophobic region of the protein (Chen and Kernohan, 1967). The possibility that stabilization of this complex is conferred through both a ligand–metal bond and hydrophobic forces has stimulated us to examine the kinetics of this reaction. Kinetic investigations have been a useful approach in coordination chemistry for understanding the mechanism of ligand–metal substitutions and similar studies should help in delineating the role played by a ligand–metal bond in the formation and stabilization of this enzyme–inhibitor complex.

While sulfonamide inhibition constants of carbonic anhydrase catalytic activity have been extensively examined, rate constants for this interaction have been reported for only a few sulfonamides and were obtained indirectly by measuring the rate of inhibition of CO_2 hydration (Kernohan, 1966; Lindskog and Thorslund, 1969). We have been able to measure the kinetics of the reaction

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{–}S$$

directly by the stop-flow method using fluorescence quenching

* From the Medical Research Council, Molecular Pharmacology Research Unit, Medical School, Hills Road, Cambridge, England. *Received February 3, 1970.*