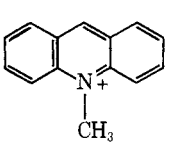
Ligand Binding Properties of Acetylcholinesterase Determined with Fluorescent Probes†

Gregory Mooser‡ and David S. Sigman*,§

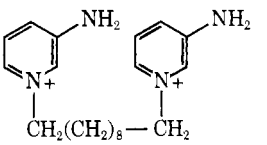
ABSTRACT: The fluorescent probes, *N*-methylacridinium ion and bis(3-aminopyridinium)-1,10-decane, have been used to monitor the ligand binding properties of acetylcholinesterase from electric eel. The displacement of the probes by cholinergic ligands is characterized by an increase of probe fluorescence as a function of displacing ligand concentration. Nonlinear least-squares regression analyses have been used to fit the data to three ligand binding models. The results indicate that *d*-tubocurarine and perhaps gallamine can bind to a peripheral ligand binding site on the enzyme which is distinct from the substrate binding site at the catalytic site. The ligand binding properties of the enzyme are very sensitive to ionic strength. At an ionic strength of 0.16 M, *d*-tubocurarine binds exclusively to the peripheral site whereas at an ionic strength of 0.002 M this receptor antagonist binds both to the catalytic site and the peripheral site. One possible conclusion from these results is that at least two conformationally distinct forms of the solubilized enzyme exist. The peripheral binding site is likely the same site which binds the second cationic group of the cholinergic ligand, decamethonium, and the fluorescent probe, bis(3-aminopyridinium)-1,10-decane. Ligand binding at the peripheral site destabilizes binding at the catalytic site. Both fluorescent probes should be useful in examining the ligand binding properties of the acetylcholine receptor protein.

The existence of a noncatalytic ligand binding site, remote from the active site on acetylcholinesterase (AcCh-esterase),[1] has been postulated to account for the results of kinetic (Changeux, 1966; Belleau *et al.*, 1970; Kitz *et al.*, 1970; Kato *et al.*, 1972a,b), affinity labeling (Belleau and Tani, 1966; Belleau and DiTullio, 1970; Meunier and Changeux, 1969), and ligand binding experiments (Kato *et al.*, 1970). While these experiments have clearly implicated the presence of more than one class of ligand binding site, on the enzyme, the relationship between the two types of sites and the possible role of the peripheral site in the cholinergic system have not been elucidated. The use of affinity ligands has been valuable in detecting the noncatalytic binding site, but this approach does not permit a thorough examination of the degree of interaction between the two sites. The interpretation of kinetic results is hindered by the preference of both sites for cationic ligands and the characteristic two-step catalytic mechanism of AcCh-esterase (Mooser and Sigman, 1972).

In order to avoid the ambiguities of a kinetic approach and the limitations imposed by affinity labels, reversible fluorescent probes of the various binding sites on AcCh-esterase were prepared. In a previous communication, we reported the properties of two such probes, *N*-methylacridinium (I) and



I



II

bis(3-aminopyridinium)-1,10-decane (II) (Mooser *et al.*, 1972). Both of these compounds are highly fluorescent in aqueous solution, but their characteristic fluorescence is greatly quenched when bound to the enzyme surface. Compound I binds stoichiometrically to the anionic subsite of the active site, and compound II, an analog of decamethonium containing two cationic functions separated by a 10 carbon alkyl chain, binds by bridging the active site and a peripheral site. Since the concentrations of the free and bound forms of I and II can be easily measured fluorometrically in a solution containing probe and AcCh-esterase, I or II can serve to monitor the binding of any ligand which affects the interaction of either of the probes with the enzyme.

In this study, we wish to report the results of analyses with AcCh-esterase based on the displacement of I and II by cholinergic inhibitors. With this technique, it has been possible to clearly define the ligand specificity of the peripheral site and quantitate the degree of its interaction with the active site. Most of the ligands examined bind exclusively at the active site of the enzyme, but *d*-tubocurarine and perhaps gallamine can bind to the peripheral site. Interestingly, changes in ionic strength of the media affect enzyme–ligand binding characteristics in a manner that cannot be strictly interpreted in terms of alterations of Coulombic forces. One possible conclusion from these results is at least two conformationally distinct forms of the solubilized enzyme exist. Since ligand binding at the peripheral site affects the catalytic activity by altering the ligand affinity of the active site, the peripheral site, in the broadest sense, may be considered, and will be referred to, as a modifier site.

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‡ National Institutes of Health Career Development Awardee. Present address: Department of Biochemistry, University of Southern California School of Dentistry, Los Angeles, Calif. 90007.

§ Alfred P. Sloan Research Fellow, 1972–1974.

[1] Abbreviations used are: AcCh acetylcholine; AcSCh, acetylthiocholine; AcCh-esterase, acetylcholinesterase; I, *N*-methylacridinium; II, bis(3-aminopyridinium)-1,10-decane; III, 7-dimethylcarbamoyl-*N*-methylquinolinium; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).

Experimental Section

Materials. *N*-Methylacridinium chloride and bis(3-aminopyridinium)-1,10-decane dichloride were prepared as previously described (Mooser *et al.*, 1972). Decamethonium bromide and gallamine triethiodide were obtained from K & K Laboratories. The chloride salts were prepared by ion exchange chromatography using a Dowex 1 chloride column, eluting with water. The chloride salts were used in all fluorescence assays. *d*-Tubocurarine chloride was purchased from K & K Laboratories and recrystallized from warm 0.1 N HCl.

Acetylcholine bromide (AcCh) was purchased from Eastman Organic Chemicals and recrystallized from ethanol. AcSCh was purchased from Sigma Chemical Co. and used without any further purification. 7-Dimethylcarbamyl-*N*-methylquinolinium iodide (III) was prepared as previously described (Mooser *et al.*, 1972).

Acetylcholinesterase from *Electrophorus electricus* was obtained from Worthington Biochemical Corp. as a partially purified lyophilized powder and used without further purification.

Methods. Buffer Media. Analyses were routinely performed in two solvent systems: one near physiological ionic strength containing 0.1 M NaCl–0.02 M $MgCl_2$–0.01 M sodium phosphate (pH 7.0); and the other at low ionic strength containing either 0.001 M sodium phosphate (pH 7.0) or 0.001 M NaCl and 1.0×10^{-4} M $MgCl_2$ (pH 7.0). Temperatures were maintained at 25°. Fluorescence measurements requiring low concentrations of I necessitated the addition of 10^{-3} M dihydroquinone.

AcCh-esterase active site titrations were performed fluorometrically by the method based on the presteady state burst reaction with III (Rosenberry and Bernhard, 1971; Mooser *et al.*, 1972).

AcCh-esterase activity was assayed with acetylcholine on a pH-Stat as previously described (Mooser *et al.*, 1972), and with AcSCh by the method of Ellman *et al.* (1961). The latter procedure was carried out in a 3-ml volume containing buffer, AcSCh, and 3.3×10^{-4} M Nbs_2 (Sigma Chemical Co.). The rate was monitored spectrophotometrically at 410 nm, and the extinction coefficient of 5-thio-2-nitrobenzene was determined from a solution of Nbs_2 in mercaptoethanol.

Enzyme assays of the presteady state rate of carbamoylation by III were performed as previously described (Mooser *et al.*, 1972).

Kinetic measurements were performed at variable substrate concentrations and analyzed by the method of Lineweaver and Burk (1934). Inhibition studies were done in the presence of one or more constant concentrations of inhibitor. The double reciprocal plots of the inhibited rates were analyzed only if they appeared consistent with competitive or mixed type inhibition. The apparent competitive inhibition constants were calculated by assuming an increase in slope of $(1 + I/K_I)$ in the presence of inhibitor (Dixon and Webb, 1964).

Fluorescent probe displacement experiments were performed by adding aliquots of a displacing ligand to a 1.0-ml solution containing buffer, AcCh-esterase, and either I or II. The increase in fluorescence as a function of the concentration of displacing ligand was measured on an Aminco-Bowman spectrophotofluorometer equipped with a Ratio photometer. Excitation and emission wavelengths were 360 and 490 nm, respectively, for I, and 330 and 410 nm for II. Concentrations of the added displacing ligand ranged from that which resulted in no probe displacement to that which gave total or near total probe displacement. Since this concentration range covered three or four orders of magnitude, several stock solutions were prepared to ensure that a single aliquot did not exceed 20 μl.

The fluorescence intensity recorded after the addition of each aliquot of displacing ligand was corrected for a small fluorescence contribution from the enzyme according to

$$I_{cor} = I_{obsd} - I_B - I_E\left(\frac{v_0}{v_0 + \Delta v}\right) \quad (1)$$

where I_{cor} is the corrected fluorescence intensity, I_{obsd} is the observed fluorescence intensity, I_B is the scattering contribution of the buffer alone, I_E is the contribution from the enzyme alone, v_0 is the initial volume, and Δv is the change in volume. The concentration of free fluorescent probe was calculated from I_{cor} by dividing by the intensity coefficient of the fluorescent probe determined in the absence of enzyme.

The free fluorescent probe concentration was plotted as a function of the common logarithm of the concentration of displacing ligand. The resultant curve was analyzed by a nonlinear least-squares regression to determine which of three enzyme ligand binding models was most consistent with the observed pattern of probe displacement.

Ligand Binding Models. Three models were considered as reasonable alternatives to describe the possible patterns of probe displacement from the enzyme. In each of the models, I and II bind at the anionic subsite of the active site (site 1), consistent with kinetic and titration data at both physiological (Mooser *et al.*, 1972) and low ionic strength (Mooser, 1972). The first of the three models (model i) only permits binding to site 1 and represents a simple competitive scheme. The other two models (models ii and iii) incorporate a peripheral modifier site (site 2) which is specific for the displacing ligand.

In the description of the models to follow, F refers to the fluorescent probe, D refers to the displacing ligand, and the subscript on D denotes binding at either the active site ($E–D_1$) or a peripheral site ($E–D_2$). The subscripts of the dissociation constants indicate the species into which a given complex dissociates.

Model i. The displacing ligand binds at the same site as the fluorescent probe (the active site or site 1); the two ligands are mutually exclusive. The following equilibria and their dissociation constants are relevant.

$$E + F \rightleftharpoons E\text{–}F$$

$$K_{E,F} = [E][F]/[E\text{–}F] \quad (2)$$

$$E + D \rightleftharpoons E\text{–}D_1$$

$$K_{E,D_1} = [E][D]/[E\text{–}D_1] \quad (3)$$

Model ii. The displacing ligand binds at a peripheral site (site 2) which results in only partial displacement of the fluorescent probe from the active site. Equilibria 2, 4, 5, and 6 are

$$E + D \rightleftharpoons E\text{–}D_2$$

$$K_{E,D_2} = \frac{[E][D]}{[E\text{–}D_2]} \quad (4)$$

$$E\text{–}F + D \rightleftharpoons E\text{–}F\text{–}D_2$$

$$K_{E\text{–}F,D_2} = \frac{[E\text{–}F][D]}{[E\text{–}F\text{–}D_2]} \quad (5)$$

$$E\text{–}D_2 + F \rightleftharpoons E\text{–}F\text{–}D_2$$

$$K_{E\text{–}D_2,F} = \frac{[E\text{–}D_2][F]}{[E\text{–}F\text{–}D_2]} \quad (6)$$

involved. $E–D_2$ is the enzyme–displacer complex with the displacing ligand occupying the peripheral site; $E–F–D_2$ is a ternary complex containing enzyme, fluorescent probe at site

1, and displacing ligand at site 2. Equation 6 is redundant since $K_{E-D_2,F} = (K_{E-F,D_2})(K_{E-F}/K_{E,D_2})$.

Model iii. The displacing ligand competes with the probe at site 1 (as in model i) and also may bind at site 2 (as in model ii). This permits the formation of the ternary complex $E-D_1-D_2$. Equations 2–8 apply. In addition to eq 6, eq 8 is redundant

$$E-D_2 + D \rightleftharpoons E-D_1-D_2$$

$$K_{E-D_2,D_1} = \frac{[E-D_2][D]}{[E-D_1-D_2]} \quad (7)$$

$$E-D_1 + D \rightleftharpoons E-D_1-D_2$$

$$K_{E-D_1,D_2} = \frac{[E-D_1][D]}{[E-D_1-D_2]} \quad (8)$$

since $K_{E-D_1,D_2} = (K_{E-D_2,D_1})(K_{E,D_2}/K_{E,D_1})$.

The explicit equations for each of the models relating the free probe concentration, $[F_f]$, to the total displacing ligand concentration, $[D_0]$, at a given total enzyme concentration, $[E_0]$, and total probe concentration, $[F_0]$, are given below for models i, ii, and iii in eq 9, 10, and 11, respectively.

$$[F_f] = \frac{-([E_0] - [F_0] + K_{E,F}(1 + [D_0]/K_{E,D_1})) + [([E_0] - [F_0] + K_{E,F}(1 + [D_0]/K_{E,D_1}))^2 + 4[F_0]K_{E,F}(1 + [D_0]/K_{E,D_1})]^{1/2}}{2} \quad (9)$$

$$[F_f] = \frac{-[([E_0] - [F_0])(1 + [D_0]/K_{E-F,D_2}) + K_{E,F}(1 + [D_0]/K_{E,D_2})] + \{[([E_0] - [F_0])(1 + [D_0]/K_{E-F,D_2}) + K_{E,F}(1 + [D_0]/K_{E,D_2})]^2 + 4[F_0]K_{E,F}(1 + [D_0]/K_{E,D_2})(1 + [D_0]/K_{E-F,D_2})\}^{1/2}}{2(1 + [D_0]/K_{E-F,D_2})} \quad (10)$$

$$[F_f] = \frac{-[([E_0] - [F_0])(1 + [D_0]/K_{E-F,D_2}) + K_{E,F}(1 + [D_0]/K_{E,D_1} + [D_0]/K_{E,D_2} + [D_0]^2/K_{E,D_2}K_{E-D_2,D_1})] + \{[([E_0] - [F_0])(1 + [D_0]/K_{E-F,D_2}) + K_{E,F}(1 + [D_0]/K_{E,D_1} + [D_0]/K_{E,D_2} + [D_0]^2/K_{E,D_2}K_{E-D_2,D_1})]^2 + 4[F_0]K_{E,F}(1 + [D_0]/K_{E-F,D_2}) \times (1 + [D_0]/K_{E,D_1} + [D_0]/K_{E,D_2} + [D_0]^2/K_{E,D_2}K_{E-D_2,D_1})\}^{1/2}}{2(1 + [D_0]/K_{E-F,D_2})} \quad (11)$$

The equations incorporate the assumption that $[D_0] \gg [E_0]$. In cases where this is not valid, the concentration of free displacer, $[D_f]$, was calculated by iteration between the appropriate equation for $[F_f]$ and the equation relating $[D_f]$ to $[D_0]$ for models i, ii, and iii given in eq 12, 13, and 14, respectively.

$$[D_f] = \frac{[D_0]}{[1 + (K_{E,F}/K_{E,D_1})([F_0] - [F_f])/[F_f]]} \quad (12)$$

$$[D_f] = \frac{-\left(K_{E-F,D_2} - [D_0] + \left[\frac{[F_0] - [F_f]}{[F_f]}\right]([F_f] + K_{E-D_2,F})\right) + \left[(K_{E-F,D_2} - [D_0] + \left[\frac{[F_0] - [F_f]}{[F_f]}\right] \times ([F_f] + K_{E-D_2,F})^2 + 4[D_0]K_{E-F,D_2}\right]^{1/2}}{2} \quad (13)$$

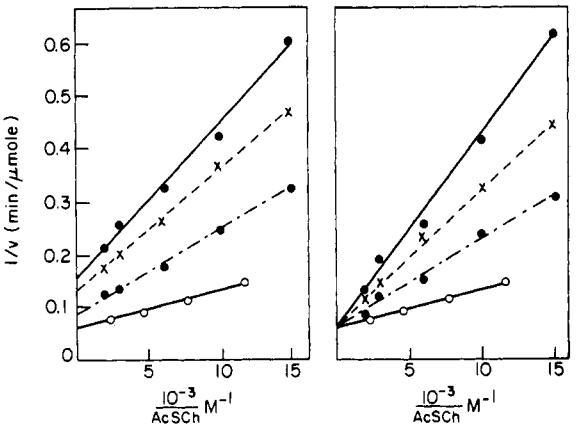

FIGURE 1: AcCh-esterase inhibition kinetics with decamethonium and gallamine. Assays were performed with AcSCh as described in the Experimental Section in a buffer containing 0.1 M NaCl–0.02 M $MgCl_2$–0.01 M phosphate (pH 7.0) at 25° with approximately 3.0×10^{-11} N AcCh-esterase. Left: decamethonium inhibition with (○—○) no decamethonium; (●- - -●) 8.3×10^{-6} M; (×- - -×) 1.7×10^{-5} M; (●—●) 2.5×10^{-5} M. Right: gallamine inhibition with (○—○) no gallamine; (●- - -●) 3.9×10^{-4} M; (×- - -×) 7.8×10^{-4} M; (●—●) 1.2×10^{-3} M.

$$[D_f] = \frac{-\left((K_{E,D_2} - [D_0]) + \left[\frac{[F_0] - [F_f]}{[F_f]}\right] \times ([F_f] + K_{E-D_2,F} + K_{E,F}K_{E-F,D_2}/K_{E,D_1})\right) + \left\{K_{E-F,D_2} - [D_0] + \left[\frac{[F_0] - [F_f]}{[F_f]}\right]([F_f] + K_{E-D_2,F} + K_{E,F}K_{E-F,D_2}/K_{E,D_1})^2 + 4[D_0]K_{E-F,D_2} \times (1 + 2K_{E-D_2,F}/K_{E-D_2,D_1})\left[\frac{[F_0] - [F_f]}{[F_f]}\right]\right\}^{1/2}}{2(1 + 2K_{E-D_2,F}/K_{E-D_2,D_1})[([F_0] - [F_f])/[F_f]]} \quad (14)$$

In the regression analysis, the total enzyme and probe concentrations were treated as independent variables so they could be varied as the volume of the initial solution increases with addition of each aliquot of displacing ligand. The enzyme–probe dissociation constant, $K_{E,F}$, was determined independently by direct titration and supplied to the program.

Results

Inhibition Kinetics. Ligand interactions with AcCh-esterase were analyzed initially by kinetic methods. This permitted the determination of apparent dissociation constants for several ligands of interest and aided in the detection of unusual kinetic patterns. Three assay systems were used, including the steady-state reactions with AcCh and AcSCh and the pre-steady-state reaction with III. The latter assay had the advantage of permitting an analysis of inhibitor effects on the acylation reaction in the absence of parameters unique to the deacylation reaction.

Assays were performed in both physiological (0.1 M NaCl–0.02 M $MgCl_2$–0.01 M sodium phosphate (pH 7.0) at 25°) and low ionic strength media (either 0.001 M sodium phosphate or 0.001 M NaCl and 2.0×10^{-4} M $MgCl_2$ at pH 7.0, 25°). The inhibition pattern observed for nearly all of the ligands tested with AcCh and AcSCh was a mixed type inhibition, except for gallamine inhibition at both physiological and low ionic strength which was purely competitive. The double reciprocal plots were linear over the range of substrate concentrations tested. Figure 1 shows a typical inhibition pattern produced by

TABLE I: Inhibition of Acetylcholine and Acetylthiocholine Hydrolysis at Physiological Ionic Strength.[a]

Inhibitor	Acetylcholine		Acetylthiocholine	
	K_I (M)[b]	Type of Inhibition	K_I (M)[b]	Type of Inhibition
I	2.2×10^{-7}	Mixed	2.0×10^{-7}	Mixed
II	6.2×10^{-8}	Mixed	4.7×10^{-8}	Mixed
Decamethonium	5.4×10^{-6}	Mixed	3.4×10^{-6}	Mixed
Gallamine	2.8×10^{-4}	Competitive	1.4×10^{-4}	Competitive
d-Tubocurarine	2.8×10^{-5}	Mixed	6.9×10^{-5}	Mixed

[a] Assays performed as described in Methods. [b] Inhibition constants were determined by double reciprocal plots at two or three separate inhibitor concentrations.

TABLE II: Inhibition of Acetylcholine and Acetylthiocholine Hydrolysis at Low Ionic Strength.[a]

Inhibitor	Acetylcholine		Acetylthiocholine	
	K_I (M)[b]	Type of Inhibition	K_I (M)[b]	Type of Inhibition
I	1.3×10^{-8}	Mixed	1.6×10^{-8}	Mixed
II	9.3×10^{-10}	Mixed	3.0×10^{-10}	Mixed
Decamethonium	3.1×10^{-8}	Mixed	2.7×10^{-8}	Mixed
Gallamine	1.9×10^{-7}	Competitive	9.5×10^{-8}	Competitive
d-Tubocurarine	1.3×10^{-6}	Mixed	6.4×10^{-6}	Mixed

[a] Assays performed as described in Methods. [b] Inhibition constants were determined from double reciprocal plots at two or three separate inhibitor concentrations.

gallamine and decamethonium in assays with AcSCh at physiological ionic strength. In Tables I and II the calculated dissociation constants and the type of inhibition observed for all of the ligands tested are reported.

Mixed type inhibition has been reported for many AcCh-esterase inhibitors and is generally attributed to the formation of an acetyl-enzyme–inhibitor complex which deacylates at a slower rate than the free acetyl-enzyme (Krupka and Laidler, 1961). Since comparable complexes do not form in the pre-steady-state reaction with III, inhibitors which exhibit mixed inhibition with AcCh and AcSCh are competitive with III. Inhibition constants determined in assays with III were consistent with those determined using AcCh and AcSCh and are reported in Table III.

TABLE III: Inhibition of the Presteady-State Reaction of 7-Dimethylcarbamoyl-*N*-methylquinolinium Hydrolysis.[a]

Inhibitor	Assay Conditions[b]	K_I (M)
I	1	3.4×10^{-7}
II	1	5.1×10^{-8}
Decamethonium	1	8.2×10^{-6}
Gallamine	1	3.2×10^{-4}
d-Tubocurarine	1	3.0×10^{-4}
Decamethonium	2	3.3×10^{-8}
Gallamine	2	5.0×10^{-8}
d-Tubocurarine	2	*c*

[a] The assays were performed as described in Methods and analyzed by double reciprocal plots at a single inhibitor concentration. [b] Condition 1 is physiological ionic strength buffer containing 0.1 M NaCl–0.02 M $MgCl_2$–0.01 M sodium phosphate (pH 7.0), 25°. Condition 2 is low ionic strength buffer containing 0.001 M sodium phosphate (pH 7.0), 25°. [c] The *d*-tubocurarine data at low ionic strength were not analyzed because the inhibition pattern was clearly not competitive.

The low ionic strength presteady-state results of III are more complex than those at physiological ionic strength. This partly results from the relatively high enzyme concentrations required to detect released 7-hydroxy-*N*-methylquinolinium in the presteady-state reaction (Rosenberry and Bernhard, 1971). Since the enzyme concentration must be greater than 10^{-8} M, the low dissociation constants of I and II as well as their interfering fluorescence prevented the use of inhibitor concentrations in a range significantly higher than the enzyme concentration, which is generally necessary for meaningful kinetic analysis. For this reason I and II were not studied as inhibitors of the reaction of III at low ionic strength. However, inhibition studies with gallamine, decamethonium and *d*-tubocurarine were performed and the results are presented in Figure 2. The double reciprocal plot produced by *d*-tubocurarine shows a pronounced deviation from linearity, and the intercept is nearly one-half the control value. The intercept in gallamine inhibition also appears displaced downward, but the significance of this relative to the experimental error is questionable.

There is a general agreement among inhibition constants for each ligand in assays with different substrates at a given ionic strength. A clear exception is the large differences observed with *d*-tubocurarine. At physiological ionic strength, for example, the measured inhibition constant for *d*-tubocurarine is 3.0×10^{-4} M using III, 2.8×10^{-5} M using AcCh, and 6.9×10^{-5} M using AcSCh. These deviations are far beyond experimental error. Subsequent studies by fluorescent probe displacement experiments have shown, as suspected,

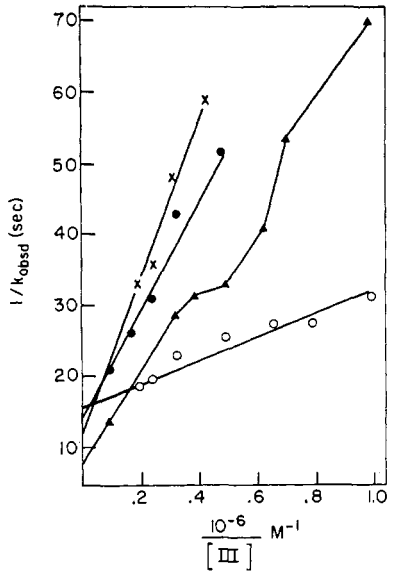

FIGURE 2: Inhibition of the presteady-state reaction of III at low ionic strength. Buffer media contained 0.001 M phosphate (pH 7.0), 25°: (○—○) no inhibitor; (▲—▲) 5×10^{-6} M *d*-tubocurarine; (●—●) 4.8×10^{-7} M decamethonium; (×—×) 4.0×10^{-7} M gallamine.

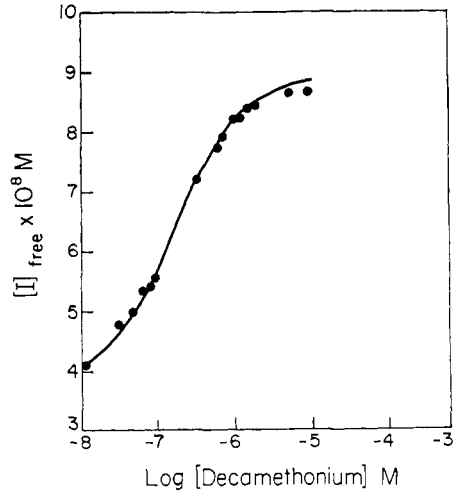

FIGURE 3: Decamethonium displacement of I at low ionic strength. Buffer contained 0.001 M phosphate–0.001 M dihydroquinone (pH 7.0) at 25°. The total AcCh-esterase concentration in the initial 1-ml solution was 9.0×10^{-8} M, and the fluorescent probe (I) concentration was 1.0×10^{-7} M. Solid dots represent experimental points and the solid line represents the best fit to model i. The best fits to models ii and iii superimpose on the line for model i.

d-tubocurarine is not purely competitive with ligand binding at the active site, but binds to a peripheral modifier site. The inhibition pattern is characteristic of partial competitive inhibition (Dixon and Webb, 1964), and the variation in inhibition constants reflects differences in inhibitor concentrations used in each assay.

Fluorescent Probe Displacement. I and II were used as fluorescent probes to further analyze the binding of the ligands studied by inhibition kinetics. With the elimination of catalytic parameters, the characteristics of the enzyme–ligand interactions could be more rigorously defined, particularly in relation to those ligands which presented unusual kinetic results.

Experiments were performed by adding aliquots of a displacing ligand to a solution containing enzyme and I or II. The initial solution was prepared with a major portion of the probe in the enzyme-bound form. The increase in free probe concentration as a function of displacing ligand concentration was followed fluorometrically and analyzed by a nonlinear least-squares regression to determine which of three enzyme ligand binding models was most consistent with the pattern of probe displacement (see Methods for a description of the three models).

A typical analysis is shown in Figure 3 for the case of decamethonium displacement of I in 0.001 M sodium phosphate at pH 7.0. The solid line represents the fit provided by all three of the models. The residual sum of the squares for the three regressions varies by less than 10% indicating each can accurately fit the data. This reflects the fact that both models ii and iii can be reduced to model i when the appropriate dissociation constants become infinite. For example, if eq 5 (K_{E-F,D_2}) and eq 6 ($K_{E-D_2,F}$) are large relative to K_{E,D_2} and $K_{E,F}$, respectively, model ii becomes mathematically indistinguishable from model i. The relative values of the dissociation constants generated from the regression using model ii for the decamethonium data indicate this is in fact the case, and the simple competitive scheme is the most valid representation of the data. Analysis with model iii results in a good theoretical fit, but a quantitation of the dissociation constants using the equation for model iii is not possible since it may reduce to two identical functions exclusively incorporating either eq 2 and 3, or eq 2 and 4. Similar results were obtained with other ligands which were strictly competitive with binding of the fluorescent probe.

In Table IV the results of the analysis of fluorescent probe displacement experiments at physiological ionic strength are reported. The displacement pattern for all of the ligands is most consistent with model i except for *d*-tubocurarine displacement of II which is best described by model ii. Figure 4 shows the best fit curves using the latter data. By inspection, the fit provided by model i is clearly unacceptable, while the

TABLE IV: Fluorescent Probe Displacement at Physiological Ionic Strength.[a]

Displacing Ligand	Fluorescent Probe	Best Fit Model[b]	Dissociation Constants[c] (M)
Decamethonium	I	i	$K_{E,D_1} = 9.9 \times 10^{-6}$
	II	i	$K_{E,D_1} = 4.9 \times 10^{-6}$
II	I	i	$K_{E,D_1} = 2.6 \times 10^{-8}$
Gallamine	I	i	$K_{E,D_1} = 2.5 \times 10^{-4}$
	II	i	$K_{E,D_1} = 1.6 \times 10^{-4}$
d-Tubocurarine	I	ii	$K_{E,D_2} = 5.6 \times 10^{-5}$
			$K_{E-F,D_2} = 3.2 \times 10^{-4}$
			$K_{E-D_2,F} = 1.5 \times 10^{-6}$
d-Tubocurarine	II	i	$K_{E,D_1} = 3.2 \times 10^{-5}$

[a] Experiments were performed as described in Methods in media containing 0.1 M NaCl–0.02 M $MgCl_2$–0.01 M sodium phosphate (pH 7.0), 25°. [b] The best fit model is one of three displacement schemes described in the Methods section which gave the lowest residual sum of the squares in a nonlinear least-squares regression when applied to the experimental data. [c] Dissociation constants specific to the best fit model generated by the regression analysis. The values of $K_{E,F}$ for I and II respectively are 2.5×10^{-7} M and 4.7×10^{-8} M.

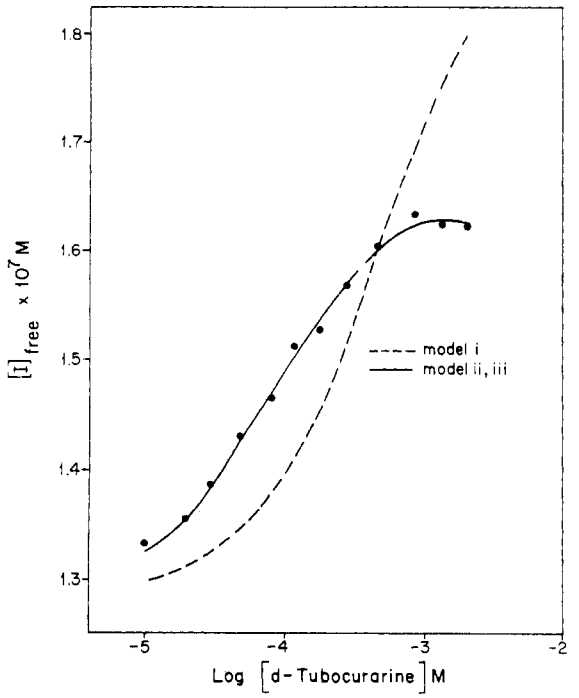

FIGURE 4: *d*-Tubocurarine displacement of I at physiologic ionic strength (μ = 0.16 M). Buffer contained 0.1 M NaCl–0.02 M $MgCl_2$, 0.01 M phosphate–0.001 M dihydroquinone (pH 7.0), 25°. Total AcCh-esterase concentration in the initial 1-ml solution was 2.2×10^{-7} N. [I] was 2.0×10^{-7} *M*. Solid dots represent experimental points. The best fits to models i, ii, and iii are indicated in the figure.

fit using model ii is excellent. The residual sum of the squares for model i is nearly 300 times greater than that for model ii. Model iii, which reduces to model ii when the dissociation constants for equilibria 3, 7, and 8 go to infinity, is equally suitable. It may be concluded from these analyses at physiological ionic strength that, according to model ii, *d*-tubocurarine does not bind to the active site, but binds to a peripheral site and may form a ternary complex with I binding at the active site. The affinity of the enzyme for I in the presence of *d*-tubocurarine decreases by a factor of 6 relative to the affinity in the absence of *d*-tubocurarine (compare $K_{E,F}$ and $K_{E-D_2,F}$).

Interestingly, *d*-tubocurarine displacement of II at physiological ionic strength is purely competitive. An interpretation of these results, which is consistent with kinetic (Changeux, 1966) and affinity labeling (Belleau and DiTullio, 1970) results, is that the peripheral *d*-tubocurarine binding site is the same site that binds the distal cationic functions of II. Therefore, the enzyme–*d*-tubocurarine–probe ternary complex is allowed with I but not with II.

The ligand binding results at low ionic strength significantly differ both quantitatively and qualitatively from those observed at physiological ionic strength (Table V). Displacement of I by either decamethonium, II, or gallamine and displacement of II by decamethonium and gallamine are consistent with model i. However, the pattern of displacement of I by *d*-tubocurarine, which at physiological ionic strength was most consistent with model ii, is best described by model iii at low ionic strength. In Figure 5 the best fit of these data by model iii has a residual sum of the squares which is 190 times less than that calculated for model i and 20 times less than that for model ii. The II displacement of *d*-tubocurarine, shown in Figure 6, is also best described by model iii with the residual sum of the squares 15 times less than either model i or model ii. Thus, under low ionic strength conditions, *d*-tubocurarine binds at both the active site and peripheral site. The binding affinity at both sites is significant. The average dissociation constant at the active site, K_{E,D_1}, is 3.1×10^{-7} M and at the peripheral site, K_{E,D_2}, is 9.9×10^{-7} M. Additionally, the dissociation constants determined from *d*-tubocurarine displacement of II indicate the enzyme–*d*-tubocurarine–II ternary complex cannot form. This is consistent with the physiological ionic strength results demonstrating the peripheral *d*-tubocurarine binding site is the same site that binds the second cationic function of II.

TABLE V: Fluorescent Probe Displacement at Low Ionic Strength.[a]

Displacing Ligand	Fluorescent Probe	Best Fit Model[b]	Dissociation Constant (M)[c]
Decamethonium	I	i	$K_{E,D_1} = 2.7 \times 10^{-8}$
	II	i	$K_{E,D_1} = 2.1 \times 10^{-8}$
II	I	i	$K_{E,D_1} = 1.8 \times 10^{-10}$
Gallamine	I	i	$K_{E,D_1} = 4.8 \times 10^{-8}$
	II	i	$K_{E,D_1} = 3.3 \times 10^{-8}$
d-Tubocurarine	I	iii	$K_{E,D_1} = 4.1 \times 10^{-7}$
			$K_{E,D_2} = 8.7 \times 10^{-7}$
			$K_{E-F,D_2} = 4.5 \times 10^{-6}$
			$K_{E-D_2,F} = 8.3 \times 10^{-8}$
			$K_{E-D_1,D_2} = 1.4 \times 10^{-4}$
			$K_{E-D_2,D_1} = 6.9 \times 10^{-5}$
	II	iii	$K_{E,D_1} = 2.1 \times 10^{-7}$
			$K_{E,D_2} = 1.1 \times 10^{-6}$
			$K_{E-F,D_2} = \infty$
			$K_{E-D_2,F} = \infty$
			$K_{E-D_1,D_2} = 4.5 \times 10^{-5}$
			$K_{E-D_2,D_1} = 8.7 \times 10^{-6}$

[a] Experiments were performed as described in Methods in media containing 0.001 M sodium phosphate (pH 7.0), 25°. [b] The best fit model is one of three displacement schemes described in the Methods section which gave the lowest residual sum of the squares in a nonlinear least-squares regression when applied to the experimental data. [c] Dissociation constants specific to the best fit model generated by the regression analysis. The values of $K_{E,F}$ for I and II respectively are 1.5×10^{-8} M and 4.7×10^{-10} M.

Discussion

The active site of AcCh-esterase is spatially and functionally divided into two subsites: the esteratic subsite which is concerned with substrate hydrolysis and incorporates the reactive serine, and the anionic subsite which imparts specificity to the reaction (Froede and Wilson, 1971). There now appears to be a distinct modifier site which, when bound, has a significant influence on catalysis and ligand affinity at the active site. Fluorescent probe displacement experiments have provided a simple method to obtain a clear picture of the AcCh-esterase ligand binding topography. Both the active site and the modifier site prefer cationic ligands, but show a significant difference in specificity beyond this preference. Most ligands bind exclusively to the active site, while *d*-tubocurarine at physiological ionic strength binds exclusively to the modifier site. Bis-quaternary ammonium compounds with a charge

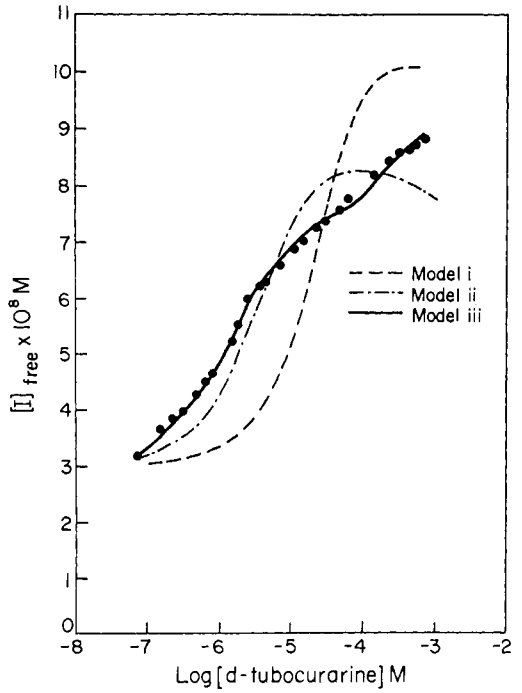

FIGURE 5: *d*-Tubocurarine displacement of I at low ionic strength. Buffer contained 0.001 M phosphate–0.001 M dihydroquinone (pH 7.0), 25°. Total AcCh-esterase concentration in the initial 1-ml solution was 1.8×10^{-7} N and [I] was 1.5×10^{-7} M. Solid dots represent experimental points. The best fits to models i, ii, and iii are indicated in the figure.

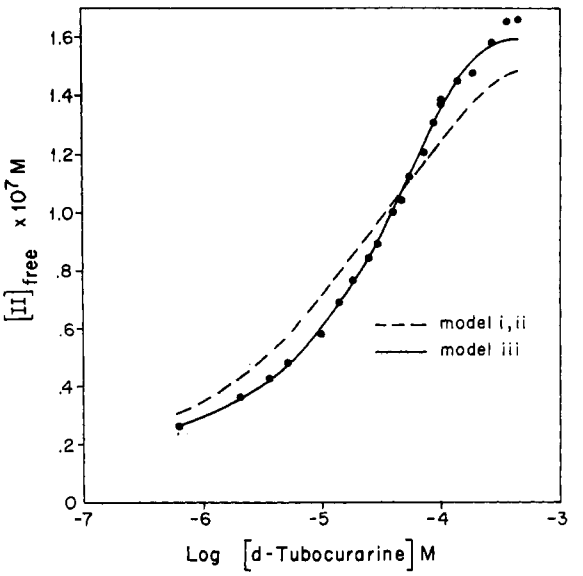

FIGURE 6: *d*-Tubocurarine displacement of II at low ionic strength (μ = 0.0016 M). Buffer contained 0.001 M phosphate (pH 7.0), 25°. Total AcCh-esterase concentration in the initial 1-ml solution was 1.8×10^{-7} N and [II] was 2.0×10^{-7} M. Solid dots represent experimental points. The best fits to models i, ii, and iii are indicated in the figure.

separation similar to decamethonium or II bind by bridging the active site and modifier site.

The presence of a peripheral modifier site on AcCh-esterase is consistent with several previously reported experimental results. Kato *et al.* (1970), using nmr, found evidence for a ternary complex composed of AcCh-esterase, atropine, and tetraethyl pyrophosphate or diisopropyl fluorophosphate. Since tetraethyl pyrophosphate and diisopropyl fluorophosphate bind to the active site, it was reasoned that atropine must be binding to a site remote from the active site. Furthermore, atropine concentrations sufficient to cause nmr spectral changes were not sufficient to inhibit AcCh hydrolysis. Similarly, Kitz *et al.* (1970), in examining the influence of reversible ligands on the rate of carbamylation and decarbamylation of AcCh-esterase by dimethylcarbamyl fluoride, found, at appropriate concentrations, gallamine did not inhibit the rate of hydrolysis of AcCh but did cause a fourfold increase in the rate of carbamylation.

The preparation of affinity labeled derivatives of AcCh-esterase has offered some of the most definitive results implicating the presence of a peripheral ligand binding site (Belleau and DiTullio, 1970). Di- and monosubstituted modification products of AcCh-esterase which have a stoichiometry of 2 mol and 1 mol of inhibitor bound per active site, respectively, can be prepared using *N,N*-dimethyl-2-phenylaziridinium. Neither the di-nor the monosubstituted enzyme binds reversible inhibitors specific for the catalytic site, but the monosubstituted enzyme does bind *d*-tubocurarine. The explanation advanced to account for this is that the disubstituted enzyme is blocked at both the active site and the modifier site, while the monosubstituted enzyme is blocked only at the active site, leaving the modifier site free to bind *d*-tubocurarine.

Using a kinetic approach, Changeux (1966) reached much the same conclusion. In studies on the *Torpedo* enzyme at low ionic strength, it was found that the inhibition pattern of gallamine and *d*-tubocurarine was consistent with a kinetic mechanism of partial competitive inhibition which requires the formation of an enzyme–substrate–inhibitor complex. As further support, it was noted that gallamine and *d*-tubocurarine antagonize the inhibition of AcCh-esterase by decamethonium. At appropriate concentrations, enzyme inhibited by decamethonium alone had less activity than enzyme inhibited by the same concentration of decamethonium with gallamine or *d*-tubocurarine added. These results are consistent with the results presented here and support an enzyme model with decamethonium bridging the active site and the peripheral site. Modifier site ligands may be totally competitive with decamethonium while only partially competitive with active site ligands.

Variations in AcCh-esterase ligand affinity with ionic strength may largely be attributed to variations in Coulombic forces. However, in order to account for ionic strength dependent changes in the *d*-tubocurarine binding pattern, additional factors such as protein structural changes or changes in enzyme sensitivity to ligand-induced conformational changes must be considered. This is apparent from the observation that *d*-tubocurarine binds strongly at both the active site and the modifier site at low ionic strength but binds only to the modifier site at high (or approximately physiological) ionic strength. There is no indication whether the physiological ionic strength or low ionic strength form of the enzyme more accurately approximates the form of the enzyme *in situ*. Kinetic differences between membrane bound and solubilized AcCh-esterase have been observed (Silman and Karlin, 1967; Webb and Johnson, 1969), but no information is available concerning the relative ligand specificity of the active site and the modifier site in a membrane fraction.

The modifier site is probably the same site which binds the second cationic function of II. The competitive displacement of II by *d*-tubocurarine at physiological ionic strength (*i.e.*, the validity of model i) indicates that the ternary complex composed

of enzyme, *d*-tubocurarine, and II cannot form. Similarly, at low ionic strength, this complex does not exist even though a ternary complex with 2 mol of *d*-tubocurarine bound does.

An estimation of the influence of the modifier site on ligand affinity at the active site is possible by examining the dissociation constants which define the ternary complexes involving *d*-tubocurarine. The average dissociation constant for the enzyme–I complex at physiological ionic strength is 2.5×10^{-7} M determined both kinetically (Tables I and III) and by direct fluorimetric titration (Mooser *et al.*, 1972). *d*-Tubocurarine bound at the modifier site destabilizes the binding of I by a factor of 6 (see $K_{E-D',F}$, Table IV). At low ionic strength there is a comparable effect. The dissociation constant for the binary enzyme–I complex is 1.5×10^{-8} M while the corresponding dissociation constant for the enzyme–I–*d*-tubocurarine ternary complex is 8.3×10^{-8} M (Table V).

These effects may reflect either steric factors, induced protein conformation changes, or both. The distance between the two sites, based on the separation between the cationic functions of II, is 14 Å or less depending on the degree of extension of the connecting alkyl chain. The relative affinities of compounds in the polymethonium series, however, suggest that decamethonium binds in a nearly extended conformation (Bergmann and Segal, 1954). The proximity of the two sites indicates steric factors may be important since *d*-tubocurarine will span most of the distance if the bulk of the molecule is directed in the region between the sites. This is supported by the observation that enzyme–I–*d*-tubocurarine is more stable by a factor of 30 with respect to the dissociation of *d*-tubocurarine from the peripheral site than the more sterically hindered enzyme–*d*-tubocurarine–*d*-tubocurarine complex (compare K_{E-F,D_2} to K_{E-D_1,D_2} in Table V).

On the other hand, ligand-induced conformational changes cannot be excluded. Results reported by Kitz and Kremzner (1968) on ORD changes of the enzyme in the presence of 3-hydroxydimethylethylammonium may indicate some ligands may exert effects at areas remote from their site of binding through induced conformational changes.

The results of gallamine binding to AcCh-esterase present an ambiguity that may not be possible to resolve with the data presently available. Fluorescent probe displacement experiments at both physiological and low ionic strength demonstrate gallamine is mutually exclusive with ligands at the active site. There are, however, two lines of kinetic evidence that suggest gallamine exerts its influence on the active site through the modifier site.

Gallamine is one of the few AcCh-esterase inhibitors that exhibits pure competitive inhibition when assayed with AcCh and AcSCh in both physiological and low ionic strength solutions (Tables I and II). All of the other inhibitors examined produce a mixed type of inhibition pattern. The noncompetitive component in the mixed inhibition is generally attributed to inhibitor binding to the acyl-enzyme intermediate. A reasonable explanation for the absence of the noncompetitive component in gallamine inhibition is that gallamine does not have a significant affinity for the acyl-enzyme intermediate. However, an equally sound explanation is that gallamine binds to the modifier site and sufficiently perturbs the structure of the active site to prevent substrate binding. Gallamine may still bind to the acyl-enzyme intermediate, but because the binding site is well removed from the active site, its presence would not affect the deacylation rate. These two alternatives cannot be distinguished kinetically. Formal competitive inhibition never can be unambiguously interpreted in terms of overlapping binding sites of the substrate and inhibitor. While this interpretation is frequently correct, the lack of a noncompetitive component in gallamine inhibition indicates the second alternative should be considered.

Gallamine binding to a site other than the active site is further supported by the inhibition of the presteady-state III reaction at low ionic strength (Figure 2). The vertical intercept of the double reciprocal plot is displaced toward the origin of the graph. While the displacement is not great, it indicates that gallamine causes an acceleration of carbamylation. Since there is no III hydrolysis in the presence of gallamine and the absence of enzyme, these results can only be explained by proposing a ternary complex composed of III, gallamine, and enzyme with III binding at the active site and gallamine at the modifier site.

These results together with previously reported results of others (Changeux, 1966; Kitz *et al.*, 1970) suggest gallamine binds to the modifier site. However, to be clearly resolved for the case of the eel enzyme, there will ultimately have to be information from another approach.

The physiological function of the peripheral modifier site on acetylcholinesterase and its relationship to the acetylcholine receptor remain to be determined. However, this modifier site may be responsible for the apparent heterogeneity of binding sites for cholinergic ligands observed in electroplax fragments and microsac preparations (Changeux and Podleski, 1968; Kasai and Changeux, 1971a,b; Eldefrawi *et al.*, 1971a,b).

Both the fluorescent probes I and II used in this study should be valuable in examining the ligand binding properties of other proteins in cholinergic synapses. Recently, Martinez-Carrion and Raftery (1973) have demonstrated that II binds to the acetylcholine receptor protein from *Torpedo californica* and that *d*-tubocurarine, acetylcholine, decamethonium, α-bungarotoxin, and calcium ion diminish its binding.

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Tyrosyl Transfer Ribonucleic Acid Synthetase from *Bacillus stearothermophilus*. Preparation and Properties of the Crystallizable Enzyme†

Gordon L. E. Koch

ABSTRACT: A procedure was developed for the purification of 100–200 mg of tyrosyl-tRNA synthetase from *Bacillus stearothermophilus*. Although thermostable, the enzyme is very sensitive to proteolysis and conditions were developed to prevent fragmentation during purification and storage. The adoption of these precautions was important for the crystallization of the enzyme (B. R. Reid, G. L. E. Koch, Y. Boulanger, B. S. Hartley, and D. M. Blow (1973), *J. Mol. Biol. 80*, 199). The enzyme is a dimer and is composed of two identical protomers with molecular weights of about 45,000 each. The enzyme contains four cysteine residues which could be useful in the preparation of heavy atom derivatives for the crystallography. Only one of these residues appears to react with 5,5′-dithiobis(2-nitrobenzoic acid) at room temperature and even this is very slow.

Aminoacyl-tRNA synthetases catalyze the esterification of amino acids to tRNA in a highly specific manner and ensure the correct translation of the genetic code. The mechanism by which these enzymes effect their bispecificity has therefore attracted a substantial amount of attention. A further source of interest in these enzymes emanates from the fact that each enzyme indulges in a very specific interaction with a well-defined polynucleotide and the system therefore lends itself readily to studies on the forces involved when proteins combine with specific nucleic acids. Since a proper understanding of either of the above processes will ultimately require knowledge of the three-dimensional structures of the enzymes, a high priority has been placed on the preparation of crystals of the enzymes suitable for study by X-ray diffraction. Before the present study three enzymes, lysyl-tRNA synthetase (Rymo *et al.*, 1970), leucyl-tRNA synthetase from yeast (Chirikjian *et al.*, 1972), and methionyl-tRNA synthetase from *Escherichia coli* (Waller *et al.*, 1971), have been crystallized in either the native or modified form.

In an attempt to overcome some of the problems experienced with enzymes from mesophiles I carried out an investigation on the aminoacyl-tRNA synthetases from the thermophile *Bacillus stearothermophilus* with the intention of preparing suitable crystalline preparations of one or more of these enzymes. This organism was chosen because it produces proteins of considerable thermal stability which can be a major asset in crystallization. Since it was not possible to decide *a priori* which protein was likely to yield suitable crystals several of the enzymes were isolated and their amenability to crystallization was tested. One of the enzymes so isolated, tyrosyl-tRNA synthetase, has yielded crystals of exceptional quality (Reid *et al.*, 1973) and we have commenced studies on the primary and tertiary structure of the protein. In this report the isolation of the crystallizable enzyme and some of its characteristics are described.

Experimental Section

Materials. Cells of *B. stearothermophilus* NCA 1503 were grown at the Microbiological Research Establishment, Porton, Wilts., U. K. tRNA was prepared by phenol extraction of cells of *B. stearothermophilus* NCA 1503 according to the procedure of Stanley and Bock (1965). Radioactive amino acids and [^{32}P]PP_i were obtained from the Radiochemical Centre, Amersham, Bucks., U. K. Activated charcoal (Noritol) was washed with distilled water and stored as a 2% solution in water. ATP, $PhCH_2SO_2F$, and Nbs_2[1] were from the

† From the Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, England. *Received January 15, 1974.*

[1] Abbreviations used are: $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).