THE ACTIVE SITE OF ACETYLCHOLIN ESTERASE PROBED BY A PHOTOCHROMIC LIGAND*

by

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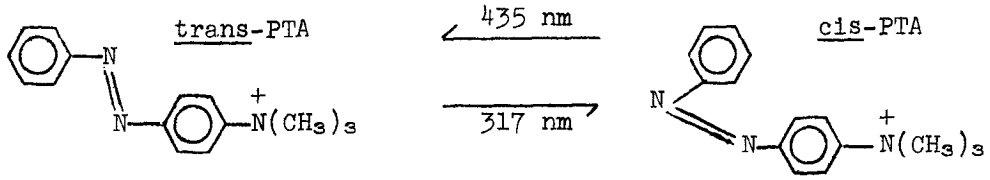
Received October 9,1972

SUMMARY:

The photochromic ligand PTA is shown to exhibit induced optical activity only if it is in the trans- form and bound to an asymmetric, ordered macromolecular matrix possessing hydrophobic binding sites. This observation can be used to probe for the existence and location of hydrophobic, ordered amino acids in the active cleft of an enzyme. It also explains the regulation of enzymic activity by reversible photo isomerization of PTA.

Some years ago Bieth, Vratsanos, Wassermann and Erlanger (1) demonstrated that the activity of acetylcholin esterase (AChE) could be photoregulated by means of the photochromic inhibitor p-phenyl azophenyl trimethyl ammonium chloride (PTA).



The spectra of the trans- and cis-PTA (Figure 1) differ appreciably so that it is possible by irradiation in the $\pi^* \leftarrow \pi$ band at 317 nm to create a photostationary state of about 80% cis. Conversely, by irradiation in the $\pi^* \leftarrow n$ band at 435 nm an 80% trans population is achieved (2).

With the ligand in the cis- form the inhibition of the enzyme is much less pronounced than it is with the ligand in the trans-form (1). Similar photochromic regulators have also been reported for the enzymic activity of chymotrypsin (3) as well as for the potential across the excitable membrane of monocellular electroplax preparations of Electrophorous electricus (4). Very recently a photo-

*Supported in part by PHS Research Grant EY-00611 and NSF Grant GB-19214

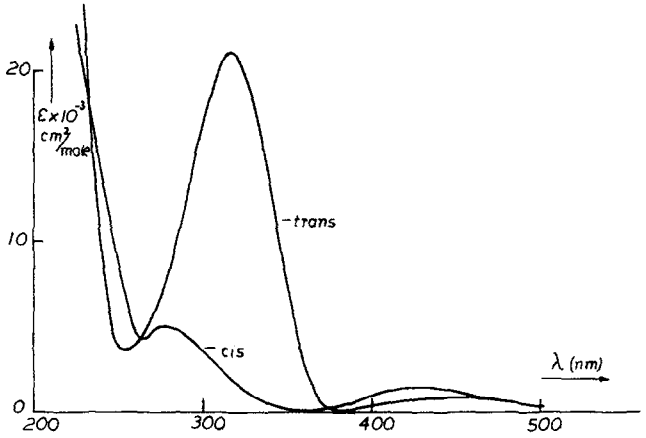


Figure 1. UV and Visible Spectra of pure trans and cis p-phenyl azo-phenyl trimethyl ammonium iodide (PTA).

chromic regulation mechanism was proposed to explain the light-stimulated or -inhibited growth of micro-organisms (5). Current photobiological thought holds that photoregulated processes both in the animal world (for example, vision) and in the plant world (for example, phototropism) all employ a molecular machinery that involves photochromic moieties embedded in a membrane matrix. Because of this perspective we have investigated the nature of the PTA - mediated regulation of AChE activity.

Figure 2 shows the molar optical rotation of AChE (from Electrophorous electricus, Worthington Corp.) in an aqueous buffer of pH = 7 taken on a Jasco-Durrum J-20 spectropolarimeter. A 1.25×10^{-3} M trans-PTA solution in the presence of 1×10^{-7} M AChE at pH = 7 exhibits two induced Cotton effects at 320 and 460 nm respectively, as shown in Figure 3. Such induced optical activity is a result of the binding of the intrinsically inactive ligand to an asymmetric matrix (6). Two additional facts are noteworthy: (1) the AChE rotation does not change upon binding of PTA so that any conformational changes in the enzyme must be very minor and (2) the $\pi^* \leftarrow n$ transition of the bound PTA is red-shifted (from 435 to 460 nm) indicating that the PTA ligand finds itself in a more hydrophobic surrounding than prior to binding (7).

Upon irradiating the trans-PTA/enzyme solution to an 80% cis photostationary state [model H3T7, General Electric, 750 Watt, medium pressure mercury arc focussed on the solution by means of a plano-convex quartz lens, employing a Corning CS-0-54 and a

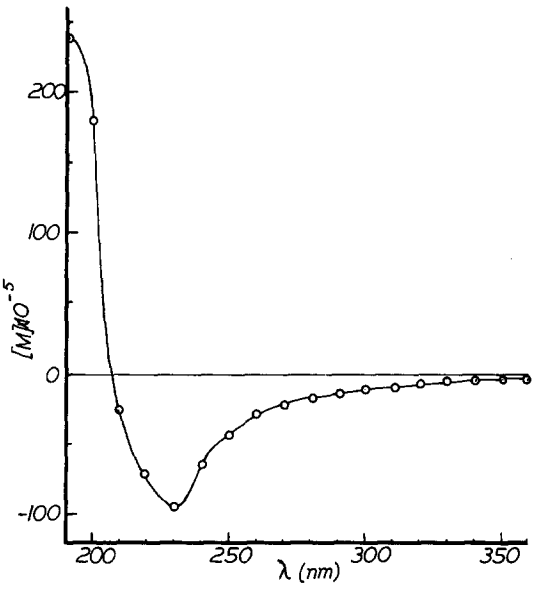


Fig. 2.

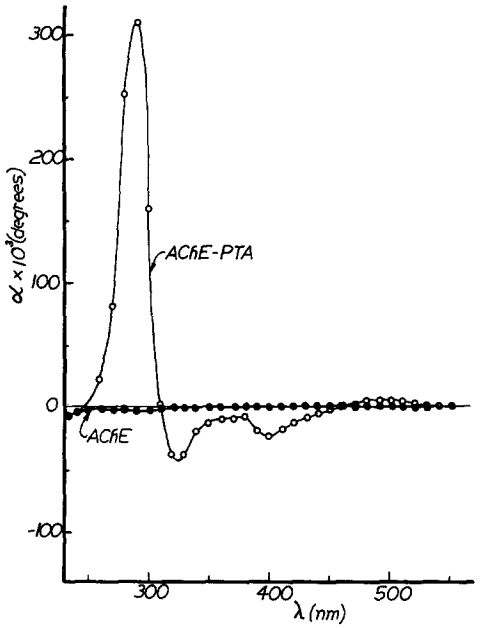


Fig. 3.

Figure 2. Molar Rotation of Acetylcholin esterase as a function of wavelength (1 mm cell, 1 x 10^{-7} M solution, pH = 7 buffer containing 0.2 M sodium phosphate, 0.1 M NaCl, 0.02 M $MgCl_2$, 0.01% gelatin and 5 x 10^{-5} M EDTA). [M] = M[α]/100 where M is the molecular weight of AChE (= 230,000) and [α] is the specific rotation defined by [α] = 100α/lc with l the optical path in decimeters, c the concentration in grams/100 ml, and α the rotation in degrees.

Figure 3. Optical Rotatory Dispersion (1 mm cell) of acetylcholin esterase and its complex with trans-PTA; 1 x 10^{-7} M AChE and 1.25 10^{-3} M trans-PTA in the same pH = 7 buffer as used for Figure 2.

Schott UG-11 filter to isolate a spectral window around 320-370 nm] the induced Cotton effects disappear completely. The induced optical activity reappears upon back photo-isomerization to an 80% trans photostationary state [35 min. irradiation with a model DWY, General Electric, 650 watt, tungsten halide lamp, focussed with a pyrex lens on the solution, employing a Corning CS-5-58 filter to isolate a spectral window at 400-480 nm]. This cycle can be repeated several times until the enzyme denatures.

The following model experiments provide insight into the nature of the binding of trans-PTA and cis-PTA, which is respon-

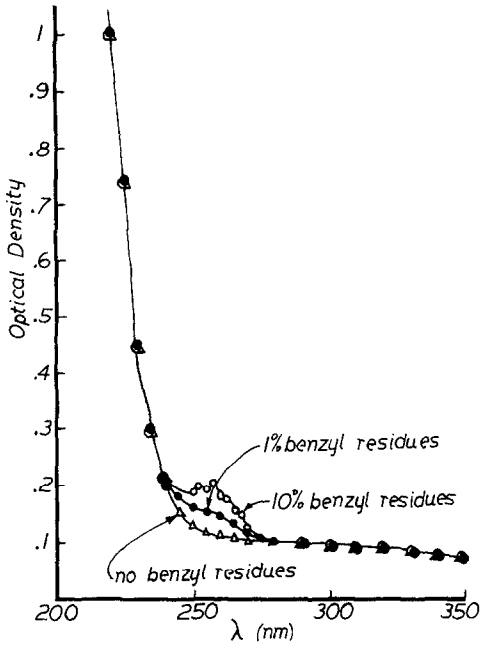


Figure 4. UV spectra in the 200-300 nm region of 4.48×10^{-3} M partially debenzylated poly-L-glutamic acid in water.

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sible for the appearance of the induced optical activity. A series of polymers were prepared by controlled debenzylation of poly-L-γ-benzyl glutamate. (Mol. weight 200,000, Sigma Chemical Corp.), leaving 10%, 1% and 0% benzyl residues on the polypeptide chain. The debenzylation was performed in dry benzene with dry HBr (8), quenching the hydrolysis reaction at different times by the addition of a large amount of water. The percentage residual benzyl group was estimated from the UV spectra in water (Figure 4) by employing the extinction coefficient at λ = 268 nm of poly L-γ-benzyl-glutamate (PGA) in dichloroethane. It was found that 1.63×10^{-3} M trans-PTA in a solution of 6.2×10^{-2} M polypeptide led to induced optical activity in the $\pi^* \leftarrow \pi$ and $\pi^* \leftarrow n$ transitions of PTA only if (1) the polypeptide was in the helical (pH = 4.5) form and (2) some residual benzyl was present on the polymer backbone (Figure 5). Again, a red shift of the $\pi^* \leftarrow n$ transition is to be noted. Also, the optical rotatory dispersion of the helical polypeptide is unaffected by the presence of trans-PTA. In the random coil conformation (at high pH) no induced Cotton effects are observed, whether one employs trans-PTA or cis-PTA.

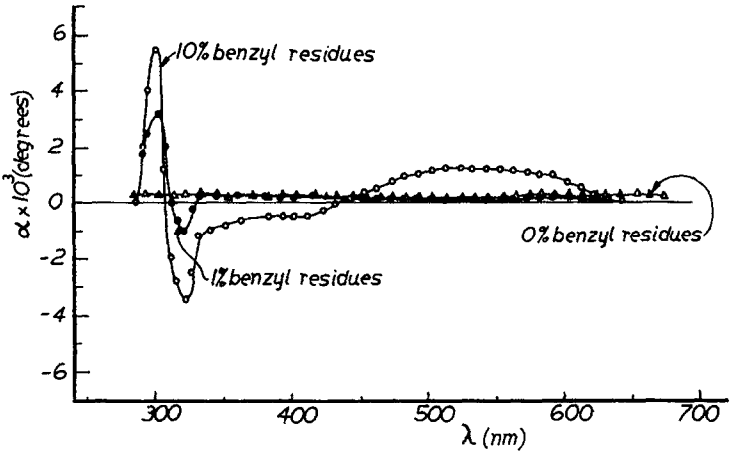

Figure 5. Optical Rotatory Dispersion of 1.63×10^{-3} M trans-PTA in the presence of 6.2×10^{-2} M solutions of various partially debenzylated poly-L-glutamic acids in the helical confirmation (pH = 4.4) (1 mm cell and corrected for the PGA rotation in this spectral region).

Interestingly, cis-PTA is converted to trans-PTA in a few minutes after the polypeptide assumes the helical conformation at low pH.

In the past it has been suggested (9) that a necessary condition for induced optical activity is the ability of the ligand to aggregate, but induced Cotton effects have later also been found in the absence of such aggregation (10). If aggregation, say to dimers, were to occur in our case, increasing the amount of trans-PTA by a factor of two should lead to a four times higher induced optical activity. This was not found to be the case: the induced optical rotation was strictly proportional to the concentration of the ligand added.

In order to establish whether hydrophobic bonding plays a role in the binding of trans-PTA to benzyl-bearing PGA, dialysis measurements were performed. Aqueous solutions of 1.3×10^{-2} M PGA were equilibrated across a semi-permeable regenerated cellulose membrane with a 1×10^{-3} M solution of trans-PTA; 1 M NaCl was present in both compartments to suppress the Donnan effect (11). The trans-PTA concentrations in both compartments were established by spectrophotometry after equilibrium was attained. The difference in the amounts of trans-PTA represents the amount bound to the polypeptide, assuming the activity coefficient of the dye to be the same in both compartments. By adjusting the pH, data were obtained

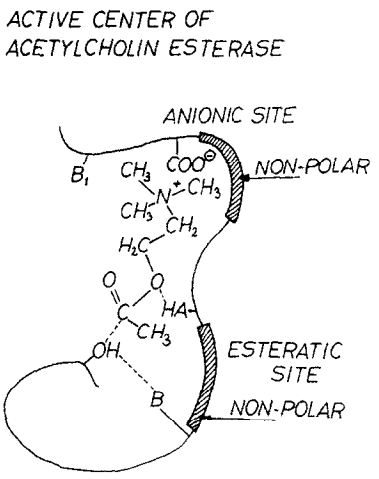

Figure 6. The active site is shown with its normal substrate in place; base B_2 and acid HA facilitate transfer of the acetyl group to the serine OH group, while the substituted ammonium ion is held at the anionic COO^- group. Base B_1 is involved in the subsequent de-acylation of the enzyme.

with PGA in the random coil as well as in the helix conformation (Table I).

Table I

Moles of trans-PTA bound per mole peptide units from equilibrium dialysis at various conformations, degrees of residual benzylation and temperatures.

	T = 25°C	T = 42°C
Random Coil PGA - 0%	2.3×10^{-3}	1.0×10^{-3}
Random Coil PGA - 1%	2.2×10^{-3}	2.5×10^{-3}
Helical PGA - 0%	1.4×10^{-3}	1.0×10^{-3}
Helical PGA - 1%	$1.3_5 \times 10^{-3}$	1.7×10^{-3}

If the binding were purely caused by electrostatic forces one would expect a decrease in binding with increasing temperature because $\Delta H < 0$ for such a process. The unbenzylated PGA-0% does indeed show this trend; of course, the extent of binding is less in the helical than in the random-coil state because the helical state carries fewer ionized carboxyl groups (lower pH). If the binding were purely caused by hydrophobic forces, i.e. driven by entropy gain in the surrounding water, ΔH would be positive and thus one would expect an increase in binding with increasing temperature. For the PGA with 1% benzyl

groups the Table shows that the hydrophobic bonding indeed dominates the electrostatic bonding.

Clearly, then, the emergence of induced optical activity in trans-PTA is a result of PTA binding to an asymmetric, ordered macromolecular matrix possessing hydrophobic binding sites. We have proven that these conditions are sufficient, and we suggest that they are also necessary. If so, the model experiments on PGA have provided us with a probe for the amino acid composition of the active site of an enzyme. If trans-PTA shows induced optical activity in its complex with an enzyme, this will indicate the presence of ordered, hydrophobic amino-acid residues in the active site; such residues would be phenylalanine, tyrosine, tryptophan and histidine.

In the case of acetylcholin esterase Rozengart (12) has recently deduced by means of enzyme kinetic studies that the active site should contain two hydrophobic regions. Figure 6 summarizes our current knowledge of the AChE active site (12,13). On the basis of the geometry of the trans-PTA the hydrophobic region close to the esteratic site should be responsible for the induced optical activity in trans-PTA.

The existence of two binding sites for trans-PTA and only one for cis-PTA explains the large difference in inhibitory effect of these two photo-isomers on the activity of acetylcholin esterase.

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