Conformational Differences between Aged and Nonaged Pyrenebutyl-Containing Organophosphoryl Conjugates of Chymotrypsin As Detected by Optical Spectroscopy†

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ABSTRACT: Homologous aged and nonaged fluorescent organophosphorus conjugates of α-chymotrypsin (Cht) were used in a comparative spectroscopic study of the conformation of their active sites, employing the pyrene group as the fluorescent probe. Steady-state fluorescence measurements showed that the quantum yield of the pyrene probe which is stoichiometrically attached to the active site is ca. 20% lower in the aged conjugate, pyrenebutyl–O–P(O)(O^-)–Cht (PBP-Cht), than in the nonaged conjugate, pyrenebutyl–O–P(O)(OC_2H_5)–Cht (PBEP-Cht). Furthermore, fluorescence decay data indicate that quenching is dynamic and is not caused by oxygen. These data, together with collisional quenching data, imply that quenching originates in an internal interaction of the fluorophore with a group within the protein. Thus, interaction of the pyrene moiety with the polypeptide chain is significantly stronger in the aged than in the nonaged conjugate, implying a different orientation of the fluorophore with respect to the protein. Circular dichroism measurements, which reflect the asymmetry of the bound pyrene in the ground state, as well as circularly polarized luminescence studies, which reflect its asymmetry in the excited state, also show that the relative configuration of the pyrene moiety and the polypeptide chain is significantly altered upon aging. Aged conjugates obtained by use of various fluorescent organophosphates [pyrenebutyl–O–P(O)Cl_2, pyrenebutyl–O–P(O)(*p*-nitrophenoxy)Cl, pyrenebutyl–O–P(O)(*p*-nitrophenoxy)$_2$] exhibit similar spectroscopic features, thus substantiating the hypothesis that instantaneous aging, by use of pyrenebutyl–O–P(O)Cl_2, and dynamic aging, by gradual removal of an aryloxy group, yield a similar product. This finding provides strong support for the formation of a P–O^- moiety in the aged conjugates, since the only expected common product of the two processes is PB–O–P(O)(O^-)–Cht. Formation of excimers of the pyrene-containing organophosphorylchymotrypsin conjugates at concentrations above 3×10^{-6} M is also reported.

Many serine hydrolases such as acetylcholinesterase (EC 3.1.1.7; AChE)[1] and chymotrypsin (EC 3.4.21.1; Cht) are inhibited by organophosphorus (OP) esters by formation of a stoichiometric (1:1) covalent conjugate with the active-site serine (Aldridge & Reiner, 1972). Such conjugates may either be reactivated by suitable nucleophiles which detach the bound phosphoryl group (Froede & Wilson, 1971) or undergo a process termed "aging", which renders them totally resistant to reactivation (Hobbiger, 1955; Berends et al., 1959). Aging may occur by either acid (Berry & Davies, 1966; Keijer et al., 1974) or base (Hovanec & Lieske, 1972; Maglothin et al., 1975) catalysis. It is usually assumed that, irrespective of its mechanism, the common denominator in the aging reaction is the detachment of one of the substituents of the phosphorus atom:

$$\mathrm{EH} + \mathrm{XP(O)(OR')R} \underset{\text{reactivation}}{\overset{\text{inhibition}\ -\mathrm{HX}}{\rightleftharpoons}} \mathrm{EP(O)(OR')R} \xrightarrow[\text{aging}]{-\mathrm{R'}} \mathrm{EP(O)(O^-)R} \quad (1)$$

where R is an alkyl, aryl, alkoxy, or aryloxy group, R′ is an alkyl or an aryl group, and X is a leaving group [e.g., F, Cl, *p*-nitrophenoxy, (dialkylamino)ethanethiol]. Only recently, however, has direct evidence been provided, from ^{31}P NMR spectroscopy, to demonstrate that the nonaged conjugate contains a triester of phosphoric acid, whereas its aged counterpart contains a diester (van der Drift et al., 1985; Grunwald et al., 1985, 1988).

Kinetic studies with neutral and negatively charged organophosphorus esters analogous to the OP–enzyme conjugates shown in eq 1 indicate that the negative charge retards nucleophilic displacement at the phosphorus atom by no more than 50–100-fold (Kirby & Younas, 1970). Thus, the electrostatic barrier imposed by the negatively charged oxygen cannot fully explain the unusual resistance to reactivation observed for aged forms of OP conjugates of AChE, Cht, or other serine hydrolases. A possible contribution to this resistance might originate in a conformational change in the enzyme occurring concomitantly with aging (Amitai et al., 1982). Fluorescence spectroscopy is a particularly sensitive technique for detecting conformational changes in proteins

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[1] Abbreviations: AChE, acetylcholinesterase; CD, circular dichroism; DFP, diisopropyl phosphorofluoridate; Cht, α-chymotrypsin; CPL, circular polarization of luminescence; HPLC, high-performance liquid chromatography; OP, organophosphoryl; PBEP, (1-pyrenebutoxy)-ethyloxyphosphoryl; PBEPF, 1-pyrenebutyl ethyl phosphorofluoridate; PBP, (1-pyrenebutoxy)hydroxyphosphoryl; PBPDC, 1-pyrenebutyl phosphorodichloridate; PB(pNP)$_2$P, 1-pyrenebutyl bis(*p*-nitrophenyl) phosphate; PB(pNP)PC, 1-pyrenebutyl *p*-nitrophenyl phosphorochloridate.

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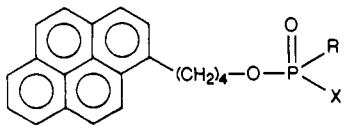
Chart I



OP ligands and conjugates	R	X
PBEPF	OC_2H_5	F
PBPDC	Cl	Cl
PB(pNP)PC	*p*-nitrophenoxy	Cl
$PB(pNP)_2P$	*p*-nitrophenoxy	*p*-nitrophenoxy
PBEP-Cht	OC_2H_5	Cht
PBP-Cht	OH	Cht

(Beechem & Brand, 1985). Berman and Taylor (1978) and Amitai et al. (1982) earlier showed that pyrene-containing OP's could be employed as probes for the active site of AChE. Furthermore, Amitai et al. (1982) were thus able to demonstrate conformational differences between aged and nonaged forms of pyrene-containing OP conjugates of AChE.

In the following, the catalytic sites of aged and nonaged pyrene-containing OP-Cht conjugates are compared by various spectroscopic techniques. For this purpose we synthesized a number of suitable pyrene-containing OP inhibitors and used them to prepare the corresponding conjugates of Cht (see Chart I).

Cht was selected since it is a well-characterized enzyme whose three-dimensional structure has been fully worked out (Blow et al., 1969; Cohen et al., 1981). Moreover, it is available in highly purified form in large quantities, permitting performance of ^{31}P NMR spectroscopy in tandem with optical spectroscopy. Indeed, ^{31}P NMR spectroscopy has already been used to study aged forms of various OP-Cht conjugates (Gorenstein & Findlay, 1976; Toia & Casida, 1979) and most recently, as mentioned above, to demonstrate that aged conjugates obtained by interaction of Cht with various OP's indeed contain a P–O^- bond (van der Drift et al., 1985; Grunwald et al., 1985, 1988). Interaction of Cht and PBEPF produces the nonaged conjugate PBEP-Cht. Interaction with the other three pyrene-containing OP's produces the aged conjugate PBP-Cht (Grunwald et al., 1985, 1988). However, whereas interaction with the dihalide PBPDC produces the aged conjugate instantaneously (Wins & Wilson, 1974; Amitai et al., 1982), interaction with the two *p*-nitrophenoxy-containing OP's PB(pNP)PC and $PB(pNP)_2P$ yields a product which ages slowly, by gradual release of the *p*-nitrophenoxy group from the OP-Cht conjugate (Grunwald et al., 1985, 1988).

The various techniques employed in this study measure spectral characteristics of the pyrene probe which are sensitive to changes in its immediate environment. Since in the stoichiometric (1:1) OP-Cht conjugates a single pyrenebutyl group is attached to the active-site serine, the spectroscopic measurements should detect putative conformational changes at the catalytic site. Thus we employ circular polarization of luminescence (CPL), which yields information concerning the asymmetry of the fluorophore in the excited state (Steinberg, 1978), as well as circular dichroism (CD), which yields similar information with respect to the ground state. In addition, steady-state and fluorescence decay measurements are utilized which are sensitive to the relative orientation of the fluorophore and the polypeptide chain. The data presented support the hypothesis that aging is accompanied by a significant conformational change at the active site. Furthermore, we demonstrate that the same aged form is obtained whether aging is instantaneous, when PBP-Cht is obtained by use of PBPDC, or whether it occurs by gradual detachment of an aryloxy group, as is the case when either PB(pNP)PC or $PB(pNP)_2P$ is used to obtain the aged conjugate.

Materials and Methods

Materials. The pyrene-containing organophosphates, PBEPF and PBPDC, were synthesized as described earlier (Amitai et al., 1982). Synthesis and characterization of PB(pNP)Cl and $PB(pNP)_2P$ will be described elsewhere (Grunwald et al., 1988).

Sodium iodide (puriss) was from Fluka, A.G. (Buchs, Switzerland); nitromethane (spectral grade) and anhydrous sodium thiosulfate (certified) were from Fisher Scientific Co. (Fairlawn, NJ); cesium chloride (suprapur) was from Merck (Darmstadt, West Germany). Other salts and buffers were Analar grade.

Preparation and Characterization of the OP-Cht Conjugates. The preparation of the various OP-Cht conjugates, the determination of their stoichiometry and of the specificity of binding, the kinetics of inhibition, and their capacity to undergo reactivation will be described in detail elsewhere (Grunwald et al., 1988). In general, the OP-Cht conjugates were obtained by dropwise addition of an acetonitrile solution (0.2–1 mM) of the appropriate fluorescent OP to a stirred aqueous solution of Cht (0.5–1 mg/mL) at room temperature. A pH of 7.4 was maintained during the inhibition reaction by addition of 0.02 N NaOH, and the fraction of organic solvent in the reaction mixture did not exceed 10%. Inhibition was followed by monitoring Cht activity according to Cunningham and Brown (1956). Residual traces of enzymic activity were abolished by adding excess DFP. The conjugates were separated from excess fluorophore by chromatography on Sephadex G-10 and then lyophilized and stored over desiccant at −20 °C until used. The stoichiometry for the aged (PBP-Cht) and nonaged (PBEP-Cht) conjugates obtained with PBPDC and PBEPF was found to be 0.97 ± 0.05 and 0.94 ± 0.03, respectively, bound fluorophore per active site. Control experiments, in which Cht was inhibited by excess DFP prior to exposure to the fluorescent OP, showed less than 3% nonspecific binding of the various fluorescent OP's. The purity of the various batches of OP-Cht conjugates was tested by HPLC, using a Mono S column (Pharmacia). Elution was performed with a NaCl gradient in 50 mM formate buffer, pH 3.5.

Spectroscopy. Absorption spectra were measured on a Zeiss PMQII spectrophotometer. Fluorescence spectra were obtained with a Hitachi Perkin-Elmer MPF 44A spectrofluorometer. In all the fluorometric measurements the pyrenebutyl probe was excited exclusively, i.e., at an excitation wavelength outside the protein absorption band.

Stern–Volmer plots of collisional quenching experiments yielded straight lines from which the quenching constants were derived by least-squares analysis.

Circular dichroism spectra were recorded with a Jasco J-500C spectropolarimeter. The absorption anisotropy factor g_{ab} is defined by $g_{ab} = (\epsilon_l - \epsilon_r)/\epsilon$, where ϵ_l and ϵ_r are the extinction coefficients for left- and right-handed circularly polarized light, respectively, and ϵ is their average.

Circularly polarized luminescence was measured with an instrument constructed at the Weizmann Institute (Steinberg & Gafni, 1972). The emission anisotropy factor g_{em} relates to the conformation of the fluorophore in the excited electronic state from which emission occurs in the same way as g_{ab} relates to the ground-state conformation (Steinberg, 1978). Thus it is defined by $g_{em} = (I_l - I_r)/(I/2)$, where I_l and I_r are the intensities of the left- and right-handed circularly polarized luminescence and I is the intensity of the total luminescence emitted.

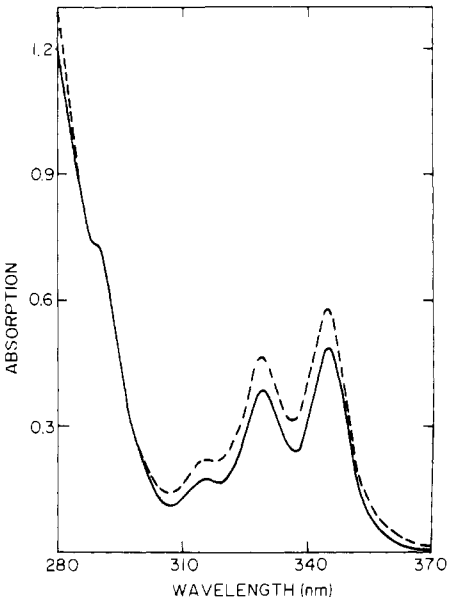


FIGURE 1: Absorption spectra of PBEP-Cht (—) and of PBP-Cht (--). The concentration of the conjugates was 4×10^{-5} M and the optical path was 0.5 cm.

Fluorescence decay measurements were performed with an instrument of the type described by Hundley et al. (1967), modified to overcome errors resulting from instrumental drift (Hazan et al., 1974). Fluorescence decay curves were analyzed by the nonlinear least-squares method (Marquardt, 1963; Grinvald & Steinberg, 1974), assuming a fluorescence decay law of the type $I(t) = \sum_i \alpha_i \exp(-t/\tau_i)$, where $I(t)$ is the decay function and α_i and τ_i are the amplitude and lifetime, respectively, of the ith component. Relative quantum yields were calculated from the fluorescence decay data according to the expression $q_r = (\sum_i \alpha_i \tau_i)/\sum_i \alpha_i$.

All spectroscopic measurements were performed in 0.1 M NaCl–0.01 M phosphate, pH 7.0, at room temperature.

Results

The homogeneity of the various pyrenebutyl OP-Cht conjugates was examined by cation-exchange HPLC. In all cases, a single major peak was obtained, with only minor contaminants (not shown). A small, unidentified component was detected, corresponding to ca. 3% of the total material. No differences were observed between the spectral characteristics of PBP-Cht or of other conjugates, before and after HPLC purification. The pyrenebutyl OP conjugates were, therefore, usually employed without the additional purification step.

The absorption spectra of the nonaged conjugate, PBEP-Cht, and of the aged conjugate, PBP-Cht, obtained with PBPDC, are shown in Figure 1. (Unless explicitly stated otherwise, the aged conjugate, PBP-Cht, referred to below, was obtained with PBPDC.) For both conjugates the pyrene absorption band is structured, with maxima at 315, 329, and 345 nm and at 315, 329, and 346 nm for the nonaged and aged conjugates, respectively. The extinction coefficient of PBP-Cht at 346 nm is ca. 15% higher than that of PBEP-Cht at 345 nm.

The CD spectra of PBEP-Cht and of PBP-Cht obtained from PBPDC and from PB(pNP)PC are presented in Figure 2. The spectra of the aged OP-Cht conjugates obtained from PBPDC and PB(pNP)PC are similar and differ markedly from that of the nonaged form, especially in the vicinity of the pyrene absorption maximum (ca. 350 nm), where the absorption anisotropy factor, g_{ab}, is much more negative for the nonaged form than for its aged counterparts, g_{ab} being -9.1×10^{-4} and -0.8×10^{-4}, respectively.

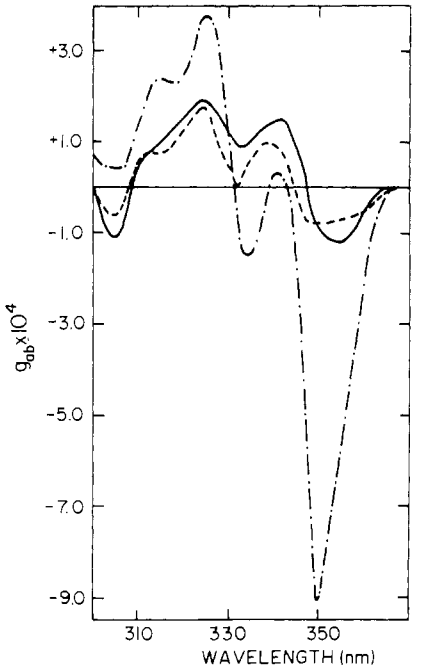


FIGURE 2: CD spectra of PBEP-Cht (-·-) and of PBP-Cht obtained with PBPDC (--) or PB(pNP)$_2$P (—). The concentration of the OP conjugates was ca. 4×10^{-5} M. The same CD spectrum was observed for PBP-Cht prepared from PBPDC, throughout the concentration range 10^{-5} to 2×10^{-4} M.

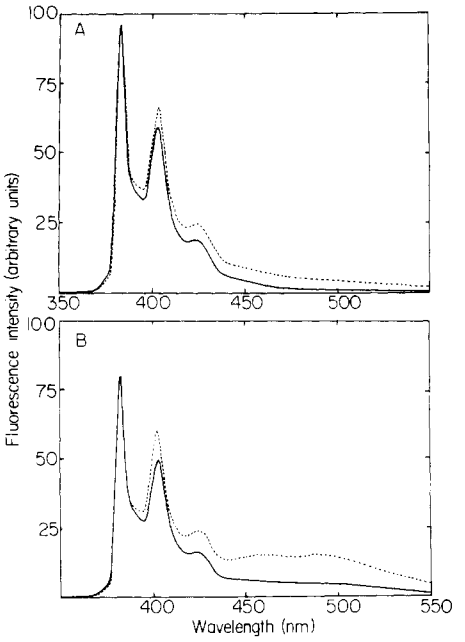


FIGURE 3: Fluorescence spectra of PBEP-Cht prepared with PBEPF (A) and of PBP-Cht prepared with PBPDC (B), at two different concentrations: 1×10^{-6} (—) and 4×10^{-5} M (--). The excitation wavelength was 345 nm. The spectrum of each conjugate is normalized at the peak to the intensity of the more dilute sample, so as to facilitate comparison.

Steady-state fluorescence measurements for PBEP-Cht and PBP-Cht are shown in Figure 3. At ca. 1×10^{-6} M both the nonaged conjugate and aged conjugates reveal similar fluorescence profiles. The quantum yield of the aged conjugate, PBP-Cht, is, however, ca. 20% lower than that of PBEP-Cht (the nonaged form). The emission spectra of the conjugates obtained by reacting Cht with either PB(pNP)PC

Table I: Collisional Quenching Constants of Free and Cht-Conjugated Pyrene-Containing Organophosphates

compound or conjugate	K_Q ($\times 10^{-2}$ M^{-1}) ± SEM for nitromethane[a]	K_Q ($\times 10^{-1}$ M^{-1}) ± SEM for I^-[a]	K_Q ($\times 10^{-1}$ M^{-1}) ± SEM for Cs^+[a]
pyrenebutanol	11.6 ± 2.3	9.4 ± 1.7	3.5 ± 0.2
PBPDC	9.3 ± 0.7	6.8 ± 0.8	1.4 ± 0.2
PBEPF	4.1 ± 1.0[b]	6.8 ± 0.8	1.2 ± 0.2
Pyr-P(O)(OC_2H_5)Cht[c]	3.2 ± 0.4	3.1 ± 0.2	0.9 ± 0.1
Pyr-P(O)(OH)Cht[c]	3.1 ± 0.3	2.0 ± 0.2	0.8 ± 0.1

[a] Results are average of at least three separate experiments. [b] 8.6 ± 0.7 after 24 h at room temperature. [c] Pyr = pyrenebutyl-O-.

or $PB(pNP)_2P$ were similar to that of the aged form, PBP-Cht, obtained by interacting PBPDC with Cht. Similar measurements at higher concentrations reveal a clear concentration dependence of the emission spectra. This is expressed primarily in the appearance of a new structureless emission band whose maximum lies at 480 nm, which is characteristic of pyrene excimers (Birks, 1970). So as to facilitate comparison, the spectrum of each conjugate is normalized at the peak to the intensity of the most dilute sample.

The accessibility to small molecules of the pyrene moiety in the aged and nonaged conjugates was examined by collisional quenching experiments. Quenching constants for I^-, Cs^+, and nitromethane, derived from Stern-Volmer plots, are presented in Table I. These results do not permit a conclusive interpretation. Nevertheless, they indicate a somewhat different accessibility of the pyrene moiety in the two conjugates to the negatively charged iodide ion.

Fluorescence decay measurements performed at different concentrations of the various OP-Cht conjugates, and collecting various parts of the emission band, are shown in Table II. The decay functions of the pyrenebutyl OP conjugates examined are not monoexponential and could be described by at least two exponents. A biexponential decay function was also observed when the samples were purified by HPLC prior to measurement. Thus the heterogeneous decay kinetics appear to reflect a heterogeneity in the interactions of the pyrene moiety with its environment, rather than a heterogeneity of the sample itself.

The molecular species revealed by the two different decay rates, which originate in the aforementioned heterogeneity of interaction of the pyrene group with its environment, differ in the spectral distribution of their fluorescence. Thus the fluorescence decay in the shorter wavelength region, 370–400 nm, displays a contribution of the long-lived component larger than its contribution to the overall fluorescence decay monitored over the whole emission band, as shown in Table II for PBP-Cht prepared from PB(pNP)PC. This implies that the fluorescence of the species with the longer lifetime is of higher energy than the emission of the short-lived species.

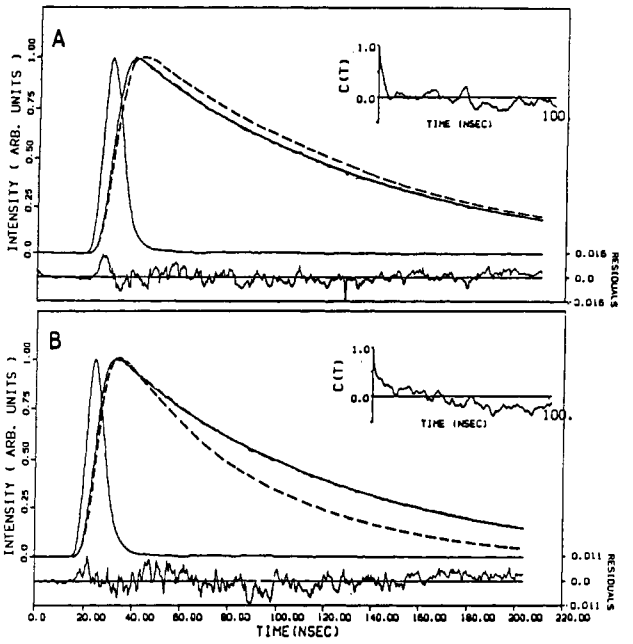


FIGURE 4: Fluorescence decay of PBEP-Cht prepared with PBEPF (A) and of PBP-Cht prepared with PBPDC (B), measured under two sets of conditions: (1) At a concentration of 1×10^{-6} M, $\lambda_{ex} = 315$ nm, $\lambda_{em} > 370$ nm (—). The decay parameters obtained for PBEP-Cht were $\tau_1 = 98.6 \pm 0.4$ ns, $\tau_2 = 6.2 \pm 0.6$ ns, $\alpha_1 = 0.105 \pm 0.001$, and $\alpha_2 = 0.018 \pm 0.001$; the corresponding decay parameters obtained for PBP-Cht were $\tau_1 = 87.7 \pm 0.3$ ns, $\tau_2 = 6.1 \pm 0.5$ ns, $\alpha_1 = 0.116 \pm 0.001$, and $\alpha_2 = 0.023 \pm 0.001$. (2) At a concentration of 2.5×10^{-5} M, $\lambda_{ex} = 315$ nm, $\lambda_{em} > 500$ nm (– –). The decay parameters obtained for PBEP-Cht were $\tau_1 = 99.9 \pm 0.3$ ns, $\tau_2 = 3.1 \pm 0.4$ ns, $\alpha_1 = 0.114 \pm 0.001$, and $\alpha_2 = -0.028 \pm 0.003$; the corresponding decay parameters obtained for PBP-Cht were $\tau_1 = 55.5 \pm 0.2$ ns, $\tau_2 = 1.7 \pm 0.3$ ns, $\alpha_1 = 0.129 \pm 0.001$, and $\alpha_2 = -0.043 \pm 0.007$. In each panel, the trace of the deviations between the theoretical and experimental decay curves for the dilute sample (1×10^{-6} M) is shown below, and the autocorrelation function for the deviations is shown in the inset at the upper right.

The fluorescence decay parameters of PBEP-Cht and PBP-Cht are shown in Figure 4. At ca. 1×10^{-6} M the nonaged conjugate, PBEP-Cht, exhibits a mean value of 5.0 ± 1.0 ns for the short-lived component, and a value of 97.5 ± 1.5 ns for the long-lived component ($n = 3$) (Figure 4A); the corresponding mean lifetimes for the aged conjugate, PBP-Cht, are 4.5 ± 1.5 and 86.0 ± 3.0 ns ($n = 3$) (Figure 4B). Similar decay parameters were obtained for PBP-Cht whether it was prepared with PBPDC, PB(pNP)PC, or $PB(pNP)_2P$ (see Table II). When the concentration is high ($>1 \times 10^{-5}$ M) and detection is focused on the red part of the spectrum, the decay function of both conjugates can be described by a biexponential expression with a negative preexponential factor (see also Table II) characteristic of an excited-state product

Table II: Fluorescence Decay Parameters of Aged and Nonaged OP-Cht Conjugates[a]

conjugate	OP used	protein concn (mg/mL)	emission band observed (nm)	α_1	τ_1 (ns)	α_2	τ_2 (ns)	RMS
PBEP-Cht	PBEPF	0.025	>370	0.105 ± 0.001	98.6 ± 0.4	0.018 ± 0.001	6.2 ± 0.6	0.0039
PBP-Cht	PBPDC	0.025	>370	0.116 ± 0.001	87.7 ± 0.3	0.023 ± 0.001	6.1 ± 0.5	0.0035
PBP-Cht	PB(pNP)PC	0.025	>370	0.110 ± 0.001	88.6 ± 0.3	0.032 ± 0.001	4.8 ± 0.4	0.0039
PBP-Cht	$PB(pNP)_2P$	0.025	>370	0.128 ± 0.001	82.3 ± 0.5	0.048 ± 0.001	3.3 ± 0.4	0.0064
PBEP-Cht	PBEPF	0.6	>500	0.114 ± 0.001	99.0 ± 0.3	-0.028 ± 0.03	3.1 ± 0.4	0.0042
PBP-Cht	PBPDC	0.6	>500	0.129 ± 0.001	55.5 ± 0.2	-0.043 ± 0.007	1.7 ± 0.3	0.0038
PBP-Cht	PB(pNP)PC	0.15	370–400	0.129 ± 0.001	84.1 ± 0.3	0.050 ± 0.001	5.4 ± 0.2	0.0034
PBP-Cht	PB(pNP)PC	0.15	>370	0.088 ± 0.001	84.8 ± 0.3	0.072 ± 0.002	6.0 ± 0.3	0.0046

[a] The results shown for each conjugate present data obtained in a single experiment which is, in each case, representative of at least three separate experiments, all of which yielded similar results. Excitation was performed with a combination of filters which transmits a narrow band whose maximum lies at 315 nm (a glass filter Schott UG11 + a chemical filter composed of 0.027 g of K_2CrO_4 + 0.1 g of Na_2CO_3 in 100 mL of water).

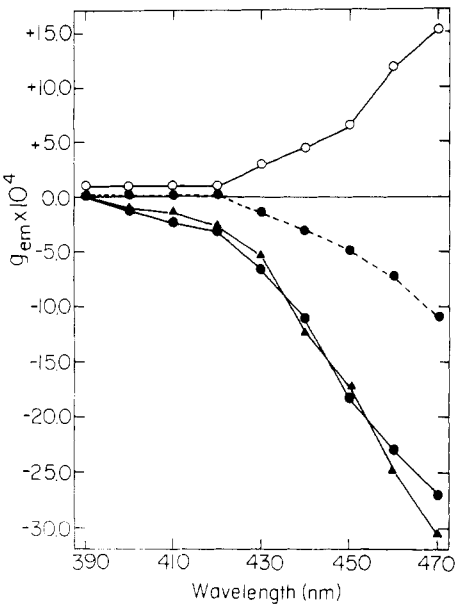


FIGURE 5: CPL spectra of PBEP-Cht prepared with PBEPF (○) and of PBP-Cht prepared with either PBPDC (●) or PB(pNP)PC (▲) at concentrations of either 4×10^{-5} (—) or 1.2×10^{-5} M (--). The excitation wavelength was 335 nm, and the experimental error in g_{em} was $\pm 0.5 \times 10^{-4}$.

(Birks, 1970), which cannot be detected at low concentrations (ca. 1×10^{-6} M).

In order to find out whether the measured lifetimes are affected by oxygen quenching, samples of the OP-Cht conjugates were examined which had previously been deoxygenated by bubbling through argon. This produced an increase of only ca. 5% in the lifetime of the long-lived component for both the aged and nonaged conjugates.

CPL spectra of the aged and nonaged conjugates are shown in Figure 5. The spectra of the aged conjugates obtained by use of PBPDC and PB(pNP)PC are very similar and differ from the spectrum of PBEP-Cht, the nonaged conjugate, both in magnitude and sign. The CPL spectra, which reflect the asymmetry of the bound pyrenebutyl probe in the excited state, exhibit an unusual concentration dependence. This, in turn, indicates the presence of an additional fluorescent species formed by a bimolecular process.

Discussion

Spectroscopic Comparison of the Aged and Nonaged Pyrenebutyl OP Conjugates of Cht. The steady-state fluorescence measurements show that the quantum yield of the pyrene in the aged conjugate, PBP-Cht, is ca. 20% lower than that in the nonaged conjugate, PBEP-Cht. Similarly, relative quantum yields, calculated from the fluorescence decay data, indicate that the relative quenching of pyrene emission in the aged conjugate, as compared to that of the nonaged conjugate, is dynamic; i.e., it occurs in the excited state (Birks, 1970). Since quenching constants derived from Stern–Volmer plots for the nonaged and aged conjugates are similar (Table I), one may infer that the accessibility of the pyrene moiety to external quenchers is roughly the same. Moreover, quenching of pyrene, in both the aged and nonaged conjugates, is not caused by oxygen (Birks et al., 1963), since deoxygenation produces only small changes in the measured lifetimes. It may, therefore, be concluded that the quenched emission of pyrene in the aged conjugate originates in an internal interaction of the fluorophore with a group within the protein. Thus, interaction of the pyrene moiety with the polypeptide chain is significantly stronger in the aged than in the nonaged conjugate; its orientation with respect to the protein must, therefore, be different in the two conjugates.

CD and CPL measurements also indicate that a conformational change takes place at the active site of the fluorescent OP-Cht conjugate concomitantly with aging. Since the absorption anisotropy factor of the aged and nonaged conjugates differs markedly in the pyrene absorption band, the ground-state conformations of their active sites must also differ markedly. The CPL measurements similarly show that the emission anisotropy factors of the aged conjugate and nonaged conjugates differ both in magnitude and in sign. The difference in magnitude is indicative of a change in the excited-state conformation of the OP-Cht conjugate upon aging, while the opposite sign implies that the induced chirality of the fluorophore within the active sites of the two conjugates is of opposite handedness. Two points should be noted: (1) The value of the emission anisotropy factor, g_{em}, is not constant across the emission band. One possible explanation of this inconstancy would be that the radiative transition (τ = ca. 100 ns) is not fully allowed (Steinberg, 1978). A second explanation would lie in an inhomogeneity of the emitting pyrenes, a possibility already suggested by the fluorescence decay data. (2) Although g_{em} depends exclusively upon the asymmetry of the emitting species, it displays a strong concentration dependence, which can most probably be ascribed to excimer formation (see below).

Comparison of Aged Conjugates Obtained by Use of Various Fluorescent Organophosphates. In all the spectroscopic measurements the properties of the aged conjugate, PBP-Cht, differed significantly from those of the nonaged conjugate, PBEP-Cht, irrespective of whether the aged conjugate was obtained with PBPDC, PB(pNP)PC, or PB-$(pNP)_2$P. Furthermore, the aged conjugates obtained with the various fluorescent OP's have rather similar spectroscopic features, thus substantiating the hypothesis that both instantaneous aging, by use of PBPDC, and dynamic aging, by gradual removal of an aryloxy group, yield a similar product. Nevertheless, both the CD and lifetime measurements exhibit small differences between the aged form obtained by use of PBPDC and that obtained by use of PB$(pNP)_2$P or PB-(pNP)PC. Thus, the aged forms obtained by use of the various organophosphoryl reagents, although very similar, are perhaps not identical.

Excimer Formation. The pyrenebutyl probe displays a tendency to form excimers (Birks, 1970). The possible formation of excimers by the OP-Cht conjugates was, therefore, examined. The structureless low-energy emission band observed for the OP-Cht conjugates at concentrations higher than 3×10^{-6} M (Figure 3) is characteristic of pyrene excimers. Furthermore, the negative preexponential factor observed in decay measurements of the OP-Cht conjugates (Table II) when monitored in the long-wavelength region, at concentrations higher than 1×10^{-5} M, is indicative of an excited-state product (Birks, 1970). Moreover, the emission anisotropy factor, g_{em}, which reflects the asymmetry of the fluorophore, whether inherent or induced, exhibits marked concentration dependence (see Figure 5), implying formation of an additional excited species. In contrast, neither the extinction coefficient nor the absorption anisotropy factor, g_{ab} (Figure 2), displays any such tendency. These findings clearly indicate that excimers, rather than dimers, are formed at concentrations higher than 3×10^{-6} M. This is, to our knowledge, the first observation of excimer formation by protein molecules in aqueous solution.

Concluding Remarks. The results presented permit the following conclusions: (1) Instantaneous aging, by use of

PBPDC, and dynamic aging, by gradual removal of an aryloxy group, yield a similar product. This finding provides strong support for the formation of a P–O^- bond in both aged conjugates, since the only expected common product of the two processes is PB–O–P(O)(O^-)–Cht; it is, furthermore, in agreement with the results of ^{31}P NMR spectroscopy studies performed in parallel (Grunwald et al., 1985, 1988). (2) The relative configuration of the pyrene moiety and the polypeptide chain is altered upon aging, due to a conformational change in the polypeptide backbone of the enzyme and/or to movement of the conjugated OP moiety.

As already mentioned, Amitai et al. (1982) reported conformational differences between aged and nonaged pyrenebutyl OP conjugates of AChE. For the fluorescent OP-AChE conjugates PBEP-AChE and PBP-AChE, the fluorescence decay lifetimes are shorter for the nonaged than for the aged conjugate, a situation opposite to that prevailing for the corresponding OP-Cht conjugates. The data for the OP-AChE conjugates were interpreted as resulting from reduced dynamic quenching by external quenchers of the pyrene in PBP-AChE due to its being less accessible than in PBEP-AChE; this interpretation was supported by collisional quenching experiments with nitromethane. Similar collisional quenching experiments with nitromethane reveal no difference in accessibility to this quencher for the two OP-Cht conjugates (Table I). Thus, while for both enzymes a conformational change is implied which may explain the common resistance to reactivation of their aged OP conjugates, the precise interactions of the fluorophore with the protein backbone appear to differ.

Aging is a process common to the OP conjugates of several serine hydrolases (Aldridge & Reiner, 1972). Furthermore, it appears to be a catalytic process (Aldridge & Reiner, 1972; Bender & Wedler, 1972) which should, therefore, involve the active-site histidine. Indeed, evidence to support this possibility has been offered both for Cht and for AChE (Toia & Casida, 1977; Beauregard et al., 1981). A plausible explanation for the observed resistance to oxime reactivation, together with the accompanying conformational change which we have demonstrated, may lie in an interaction between the negative charge of the P–O^- bond produced by aging (van der Drift et al., 1985; Grunwald et al., 1985, 1988) and the imidazole group of the active-site histidine—His-57 in the case of Cht (Blow et al., 1969). Such an interaction might stabilize the active-site region of the aged conjugate. In fact, Masson and Goasdoue (1986) have recently suggested the occurrence of such an aging-induced stabilization in OP conjugates of serum cholinesterase, on the basis of measurements of their resistance to unfolding by urea.

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