

Refined Crystal Structures of “Aged” and “Non-aged” Organophosphoryl Conjugates of γ -Chymotrypsin

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“Aged” organophosphoryl conjugates of serine hydrolases differ from the corresponding “non-aged” conjugates in their striking resistance to nucleophilic reactivation. The refined X-ray structures of “aged” and “non-aged” organophosphoryl conjugates of γ -chymotrypsin were compared in order to understand the molecular basis for this resistance of “aged” conjugates. “Aged” and “non-aged” crystalline organophosphoryl- γ -chymotrypsin conjugates were obtained by prolonged soaking of native γ -chymotrypsin crystals with appropriate organophosphates. Thus, a representative “non-aged” conjugate, diethylphosphoryl- γ -chymotrypsin, was obtained by soaking native crystals with paraoxon (diethyl-*p*-nitrophenyl phosphate), and a closely related “aged” conjugate, monoisopropyl- γ -chymotrypsin, was obtained by soaking with diisopropylphosphorofluoridate. In both crystalline conjugates, the refined structures clearly reveal a high occupancy of the active site by the appropriate organophosphoryl moiety within covalent bonding distance of Ser195 O'. Whereas in the “non-aged” conjugate both ethyl groups can be visualized clearly, in the putative “aged” conjugate, as expected, only one isopropyl group is present. There is virtually no difference between the “aged” and “non-aged” conjugates either with respect to the conformation of the polypeptide backbone as a whole or with respect to the positioning of the side-chains within the active site. In the “aged” conjugate, however, close proximity (2.6 Å) of the negatively charged phosphate oxygen atom of the dealkylated organophosphoryl group to His57 N^{e2} indicates the presence of a salt bridge between these two moieties. In contrast, in the “non-aged” conjugate the DEP moiety retains its two alkyl groups; thus, lacking a negative oxygen atom, it does not enter into such a charge-charge interaction and its nearest oxygen atom is 3.6 Å away from His57 N^{e2}. It is suggested that steric constraints imposed by the salt bridge in the “aged” conjugate lie at the basis of its resistance to reactivation.

Keywords: protein crystallography; aging; organophosphates; chymotrypsin; serine hydrolase

1. Introduction

Many serine hydrolases such as chymotrypsin (Cht†) and acetylcholinesterase are inhibited by organophosphate (OP) esters (Aldridge & Reiner, 1972). Inhibition is achieved by formation of a stoichiometric (1:1) covalent conjugate with the active site serine residue. Phosphorylated serine hydrolases may be reactivated effectively by

various nucleophiles (e.g. quaternary oximes) that detach the bound OP moiety from the serine hydroxyl group. In addition, the conjugate may undergo a dealkylation reaction, commonly termed “aging”, which converts the inhibited enzyme into a non-reactivatable form (Berends *et al.*, 1959). The “aging” process is particularly pronounced for conjugates in which the OP moiety contains a secondary alkyl group such as isopropyl or pinacolyl (Aldridge & Reiner, 1972). The loss of an alkyl group from the phosphoryl moiety of certain OP-containing serine hydrolases has been demonstrated, as predicted, to introduce a negative charge into the active site of the inhibited enzyme (van der Drift *et al.*, 1985; Grunwald *et al.*, 1989). It was

† Abbreviations used: Cht, chymotrypsin; OP, organophosphate; n.m.r., nuclear magnetic resonance; DFP, diisopropyl phosphorofluoridate; DEP, diethyl phosphoryl; MIP, monoisopropyl; MEP, monoethylphosphoryl; DIP, diisopropylphosphoryl; r.m.s., root-mean-square.

pointed out, however, that it is unlikely that the electrostatic effect of the negative charge assumed to be present in the "aged" enzyme is alone responsible for the unusual resistance to reactivation (Amitai *et al.*, 1982).

Various physicochemical techniques, such as electron spin resonance (Morrisett *et al.*, 1969), nuclear magnetic resonance (n.m.r.; Gorenstein & Findlay, 1976; van der Drift *et al.*, 1985) and fluorescence spectroscopy (Berman & Taylor, 1978; Amitai *et al.*, 1982), as well as neutron diffraction (Kossiakoff & Spencer, 1980, 1981) and X-ray crystallography (Stroud *et al.*, 1974), have been used to study the structure of OP-conjugates of serine hydrolases. Sigler & Skinner (1963) were the first to obtain a crystalline OP derivative of chymotrypsin by soaking diisopropylphosphorofluoridate (DFP) into crystals of γ -chymotrypsin (γ -Cht); they did not, however, report a detailed structure. Stroud *et al.* (1974) reported the structure, to 2.7 Å (1 Å = 0.1 nm) resolution, of a conjugate obtained by treating trypsin with DFP. This conjugate was subsequently shown to be monoisopropyl-trypsin (Kossiakoff & Spencer, 1980). No detailed comparison has been reported of analogous "aged" and "non-aged" OP conjugates of a single serine hydrolase that might permit examination and comparison of the two forms at atomic level.

Cht has been especially valuable in studying structural differences between "aged" and "non-aged" conjugates using ^{31}P n.m.r. and optical spectroscopy (van der Drift *et al.*, 1985; Grunwald *et al.*, 1989; Steinberg *et al.*, 1989). Here, we report the refined crystal structures of a "non-aged" diethylphosphoryl conjugate of γ -Cht (DEP- γ -Cht) and of the corresponding "aged" monoisopropyl conjugate (MIP- γ -Cht), obtained by soaking the appropriate OPs, paraoxon and DFP, respectively, into crystals of native γ -Cht. The structures are compared to each other and to that of the native enzyme.

2. Materials and Methods

Crystals of γ -Cht were grown following the procedure of Cohen *et al.* (1981) from twice crystallized and lyophilized γ -Cht (Sigma no. C-4754) at pH 5.6. A "non-aged" conjugate of γ -Cht with paraoxon was obtained by soaking the native crystals in a solution of 0.4 mM-paraoxon (diethyl-*p*-nitrophenylphosphate), 65% saturated $(\text{NH}_4)_2\text{SO}_4$, 0.4% (w/v) dioxane and 0.01 M-sodium cacodylate buffer (pH 5.6) for 1 month. In order to check the binding of paraoxon to the protein, the crystals were dissolved in 2.5 mM-HCl and the protein content (Bradford, 1976) and activity of the enzyme (DelMar *et al.*, 1979) were measured. The "non-aged" conjugate DEP- γ -Cht ($(\text{C}_2\text{H}_5\text{O})_2\text{P}(\text{O})\text{-Cht}$) lost 83% of its activity. An "aged" OP conjugate of γ -Cht was obtained by soaking the native crystals in a solution of 10 mM-DFP, 65% saturated $(\text{NH}_4)_2\text{SO}_4$, 2% (w/v) acetonitrile and 0.02 M-sodium cacodylate buffer (pH 4.0) for 10 weeks to yield MIP- γ -Cht ($\text{iC}_3\text{H}_7\text{OP}(\text{O})(\text{O}^-)\text{-Cht}$). A lower pH value for soaking was chosen so as to enhance the rate of "aging" (Aldridge & Reiner, 1972). In order to check the binding of the inhibitor to the protein, the crystals were analyzed as above, and this latter γ -Cht conjugate was shown to

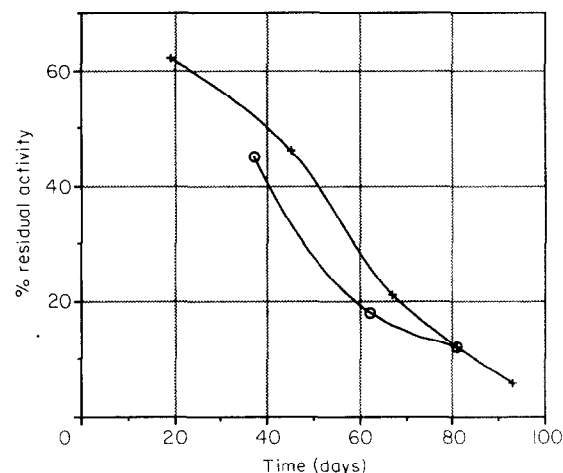


Figure 1. Inhibition of activity of "aged" crystalline γ -Cht conjugates as a function of soaking time in solution containing the appropriate organophosphate. (O), MIP- γ -Cht activity; (+), MEP- γ -Cht activity.

have lost 89% of its initial activity. Another "aged" conjugate, monoethylphosphoryl- γ -Cht ($\text{C}_2\text{H}_5\text{OP}(\text{O})(\text{O}^-)\text{-Cht}$; MEP- γ -Cht) was obtained by soaking the native crystals under the same conditions as above, but using 10 mM-ethyl isopropyl phosphorofluoridate as the OP. The synthesis and characterization of this novel OP will be reported elsewhere (Y. Ashani *et al.*, unpublished results). The crystals were soaked for 3 months and were found to be 95% inhibited. The low pH value chosen, so as to enhance "aging", may have been responsible for the slow rate of inhibition of the crystalline enzyme by these 2 latter organophosphates, the time courses for which are shown in Fig. 1.

The X-ray intensity data for the "non-aged" conjugate, DEP- γ -Cht, were collected on a Rigaku AFC5-R rotating anode diffractometer at 90 K using the cryotemperature attachment developed by Hope (1988). A complete set of 1.9 Å data was collected from a single crystal. The data show practically no decay in intensity as monitored by 3 standard reflections, spread in reciprocal space, that were remeasured every 150 reflections. Hence, no decay correction was applied. The crystal, which was mounted in a small droplet of epoxy resin, was not enclosed in a capillary and needed only a small absorption correction (maximum correction 11%), which was applied to the intensity data. The data were processed with the PROTEIN package (Steigemann, 1974). The X-ray intensity data of the "aged" conjugate, MIP- γ -Cht, were collected on a Siemens/Xentronics area detector at room temperature. The 2.1 Å data were collected from 2 crystals and processed with the XENGEN package (Howard *et al.*, 1987), with R_{merge} of 6.2% between them. The data for the 2nd "aged" conjugate, MEP- γ -Cht, were collected to 1.9 Å resolution, from a single crystal, at 90 K using the Rigaku AFC5-R diffractometer. This crystal diffracted less well, with only 78.8% of the reflections having $I > 1\sigma$ compared to 92.5% and 94.8%, respectively, for the above-mentioned "non-aged" and "aged" conjugates' data sets. Table 1 presents a summary of the crystalline conjugate preparations employed and of the conditions of data collection.

The structures were refined using the modified restrained-parameter least-square program PROLSQ (Hendrickson, 1985). The version of the program used for

Table 1
Crystal data for "non-aged" and "aged" conjugates of γ -Cht

	DEP- γ -Cht	MIP- γ -Cht	MEP- γ -Cht
Soaking conditions			
Compound	paraoxon ^a	DFP ^b	EIPF ^c
Concentration (mM)	0.4	10	10
pH	5.6	4.0	4.0
Time (weeks)	4	10	12
Inhibition (%)	83	89	85
X-ray data collection			
Temperature	90 K	room temp.	90 K
Unit cell dimensions			
<i>a</i>	69.0	69.9	68.9
<i>c</i>	95.3	96.7	95.2
Collection mode	Diffractometer	Area detector	Diffractometer
Resolution (Å)	1.9	2.1	1.9
No. of reflections measured	17,990	31,167	30,368
Theoretical no. of unique reflections	16,854	12,339	16,854
No. of reflections used for refinement	16,171 ^d	11,120 ^e	13,465 ^f
No. of crystals used for data collection	1	2	1
R_{sym}	0.025 ^g	0.068 ^h	0.107 ^b
Length of data collection (days)	5	2	8

^a Diethyl p-nitrophenylphosphate.

^b Diisopropyl phosphorofluoridate.

^c Ethyl isopropyl phosphorofluoridate.

^d $F > 0.0$.

^e $F > 3\sigma$.

^f $I > 2\sigma$.

^g $\sum |F - \langle F \rangle| / \sum \langle F \rangle$.

^h $\sum |I - \langle I \rangle| / \sum \langle I \rangle$.

these studies incorporates a fast Fourier algorithm to speed up calculations (Finzel, 1987) and it restrains intermolecular contacts (Sheriff, 1987). The DEP- γ -Cht was refined starting from the room-temperature 1.9 Å refined γ -Cht co-ordinates and temperature factors of Cohen *et al.* (1981), with no solvent molecules included. Initially, all reflections with $F > 3\sigma$ at a resolution range of 6 to 1.9 Å were used in the refinement (12,955 reflections); in the final stages, 16,171 reflections with $F > 0$ were included (out of a possible 16,854 theoretical reflections). By iterating between refinement cycles and fitting the structure to electron density maps with coefficients of $(2F_o - F_c)$ and $(F_o - F_c)$ on the interactive graphics system (Evans & Sutherland PS390) using the program FRODO (Jones, 1978; Pflugrath *et al.*, 1984), the model was fitted to the density.

The refinement of the "aged" structure, MIP- γ -Cht, started from the low-temperature 1.6 Å refined γ -Cht co-ordinates and temperature factors (Harel *et al.*, 1991) with 4 sulfate ions, but no water molecules, included. The refinement was carried out as above, at resolution range of 6 to 2.1 Å, using 11,120 reflections with $F > 3\sigma$ (out of a possible 12,339 theoretical reflections). The refinement of the second "aged" structure, MEP- γ -Cht, started from the low-temperature 1.6 Å refined γ -Cht structure, as above, with no solvent molecules included. It was stopped at an R -factor of 23.9% and the electron density showed that an "aged" conjugate had been obtained (see Results).

Refined atomic parameters and the structure factor data for the DEP- γ -Cht (the "non-aged" conjugate) and MIP- γ -Cht (the "aged" conjugate) will be deposited with the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973, U.S.A.

3. Results

(a) Structure refinement

At the initial stages of the refinement of DEP- γ -Cht, the highest positive density peak in the $(F_o - F_c)$ map was found in the active site region. The structure of the DEP part in the crystal structure of silver diethylphosphate, determined by Hazel & Collin (1972), could be fitted well to this density. The structure of DEP- γ -Cht was refined to an R -factor of 18.7% with the DEP group, 278 water molecules and three sulfate ions included.

At the initial stages of refinement of the "aged" conjugate, MIP- γ -Cht, the highest positive density peak in the $(F_o - F_c)$ map was located in the active site region. The density showed clearly that the diisopropylphosphoryl moiety (DIP) had lost one of its secondary carbon groups, leaving MIP bound to the γ -Cht. The structure of the DIP part in the crystal structure of diisopropyl-(2,3,4,5,-tetraphenyl-cyclopenta-1,4-dienyl)-phosphate, determined by Krishnanachari & Jacobson (1979) could be used to model this density. The structure was refined to an R -factor of 18.7% with the MIP group, 102 water molecules and two sulfate ions included. The second "aged" conjugate, MEP- γ -Cht, obtained by use of ethyl isopropylphosphorofluoridate, was refined until the electron density in the active site showed clearly that the isopropyl moiety of the OP was absent and that only the ethyl moiety could be discerned in the covalently bound OP. At this stage, refinement was discontinued, since it was felt that



Figure 2. Overlap of C^α chain tracings of "aged" MIP- γ -Cht (continuous line) and "non-aged" DEP- γ -Cht (broken line). The bound OPs and the active site side-chains of Ser195 and His57 are in bold lines.

no new information could be obtained from a similar fully refined "aged" structure. The resulting parameters for the two full refinements are shown in Table 2.

(b) *Binding in "aged" versus "non-aged" conjugates*

The conformation of γ -Cht in the "aged" and "non-aged" conjugates is very similar; the root-mean-square deviation in the C^α positions between the two protein conjugate molecules is 0.29 Å (see Fig. 2). The largest difference is found in the outside loop, residues 203 to 205, which shifts by about 1 Å. This shift may be ascribed to the difference between the crystal structures at low temperature *versus* room temperature (Harel *et al.*, 1991). In order to compare the conjugated Cht conformation to a "native" conformation, we used the refined structure of α -chymotrypsin (α -Cht) at 1.68 Å resolution (Tsukada & Blow, 1985), where the active site is free, since the crystalline γ -Cht is in the form of an enzyme-peptide complex (Dixon & Matthews, 1989; Harel *et al.*, 1991). The largest difference between the C^α positions of α -Cht and those of either DEP- γ -Cht or of MIP- γ -Cht was found in an outside loop, residues 75 to 79. This difference may be

ascribed to the different packing of α -Cht and γ -Cht in the two crystal forms. The conformation of this loop in γ -Cht is restricted by its interactions with a depression in a symmetry-related molecule. The interactions involve two hydrogen bonds between Ser76 O^γ and Asn236 $N^{\delta 2}(\text{sym})$ (2.81 Å) and between Ser76 O and Ala126 $N(\text{sym})$ (2.77 Å). In the dimer of α -Cht, residue 76 is also involved in hydrogen bonding with molecules related by crystal symmetry. It forms one hydrogen bond between Ser76 O (molecule A) and Thr174 $O^{\gamma 1}$ (molecule A sym) and two bonds between Ser76 O and O^γ (molecule B) and Asn167 $N^{\delta 2}$ (molecule B sym; Tsukada & Blow, 1985). However, the surfaces which interact with loops 75–79 in γ -Cht are not concave and the loops are freer to assume any conformation. When this loop is omitted from the comparison of C^α positions, the r.m.s. deviation between α -Cht and DEP- γ -Cht drops from 0.55 Å to 0.42 Å (as α -Cht data were collected at room temperature and, once more, the largest difference is found in the position of loop 203–205). The C^α r.m.s. deviation between α -Cht and MIP- γ -Cht drops from 0.55 Å to 0.44 Å when loop 75–79 is omitted. The similarity between the various Cht structures is evident also in the conformations of residues Ser195, His57 and

Table 2
Results of PROLSQ refinement of "non-aged" and "aged" conjugates of γ -Cht

	DEP- γ -Cht	MIP- γ -Cht
R-factor	0.187	0.187
Resolution (Å)	6-1.9	6-2.1
No. of water molecules	278	102
No. of sulfate ions	3	2
<i>r.m.s. deviation from restraints (σ target values in refinement)</i>		
Bond length (Å)	0.016 (0.025)	0.016 (0.025)
Bond angle distance (Å)	0.031 (0.035)	0.027 (0.031)
Planar 1-4 distance (Å)	0.036 (0.05)	0.035 (0.05)
Plane restraints	0.011 (0.02)	0.010 (0.02)
Chiral-center restraints	0.147 (0.15)	0.120 (0.15)
Non-bonded contact restraints		
Single torsion contacts	0.181 (0.5)	0.183 (0.5)
Multiple torsion contacts	0.206 (0.5)	0.201 (0.5)
Possible hydrogen bonds	0.265 (0.5)	0.215 (0.5)
Conformational torsion planar angle ω (deg.)	2.0 (2.8)	1.7 (2.8)

Table 3
Geometry of charge-relay system in various chymotrypsin structures

	DEP- γ -Cht	MIP- γ -Cht	γ -Cht ^a	α -Cht ^b
A. Hydrogen bonds (Å)				
Ser195 O ^γ -His57 N ^{ε2}	3.16	3.09	2.99	2.78
Asp102 O ^{δ2} -His57 N ^{δ1}	2.82	2.79	2.70	2.62
Asp102 O ^{δ1} -His57 N	2.87	2.82	2.96	2.84
Asp102 O ^{δ1} -Ala56 N	2.73	2.99	2.82	2.77
B. Torsion angles (deg.)				
Ser195 χ_1	-72	-71	-77	-83
His57 χ_1	89	84	84	79
His57 χ_2	-98	-93	-105	-94
Asp102 χ_1	-173	-167	-168	-167
Asp102 χ_2	-162	-163	-165	-166

^a γ -Cht at 1.6 Å containing a polypeptide in the active site (Harel *et al.*, 1991).

^b α -Cht at 1.67 Å average of 2 molecules in the dimer (Tsukada & Blow, 1985).

Asp102, which comprise the charge-relay system of the active site (Blow, 1976), as shown in Table 3. The OP moieties in both the "aged" and the "non-aged" conjugates are seen to be bound covalently to Ser195 O^γ in the active site, based on the interatomic distances shown in Table 4. The Ile16 N is hydrogen bonded to Asp194 O^{δ1} in both conjugates, as is the case for other activated Cht species (Steitz & Shulman, 1982).

It is clear from the electron density maps that, upon soaking DFP into γ -Cht crystals, one of the isopropyl groups is lost, resulting in the "aged" MIP- γ -Cht conjugate. The resulting close distance (2.6 Å) between the negatively charged phosphoryl oxygen atom and His57 imidazole N^{ε2} implies a strong charge-charge interaction (Fig. 3; 2.9 Å in the partially refined MEP- γ -Cht). In contrast, in the "non-aged" conjugate, the DEP moiety retains its two alkyl groups; thus, lacking a negatively charged oxygen atom, it does not enter into such a charge-charge interaction, and its nearest oxygen atom is 3.6 Å away from His57 N^{ε2} (Fig. 4). In both conjugates the uncharged phosphate oxygen atom is locked in the "oxyanion hole" as proposed by

Henderson (1970) and by Robillard *et al.* (1972), and is stabilized by forming two hydrogen bonds to Ser195 N and Gly193 N. The carbon atoms of the alkyl moieties of both conjugates can form hydrophobic interactions with residues 191 and 215, which are part of the hydrophobic specificity pocket of Cht (Blow, 1976).

Table 4
Bonding and short contact distances (Å) between the OP groups and surrounding residues of γ -Cht in OP- γ -Cht conjugates

Atom 1 . . . atom 2	Short contact distances (Å)	
	DEP- γ -Cht	MIP- γ -Cht
P . . . Ser195 O ^γ	1.54	1.55
O ^a . . . Gly193 N	2.83	2.94
O ^a . . . Ser195 N	2.93	3.18
O . . . His57 N ^{ε2}	3.58 ^b	2.60 ^c

^a Uncharged oxygen atom.

^b Ethoxy oxygen atom.

^c Charged oxygen atom.

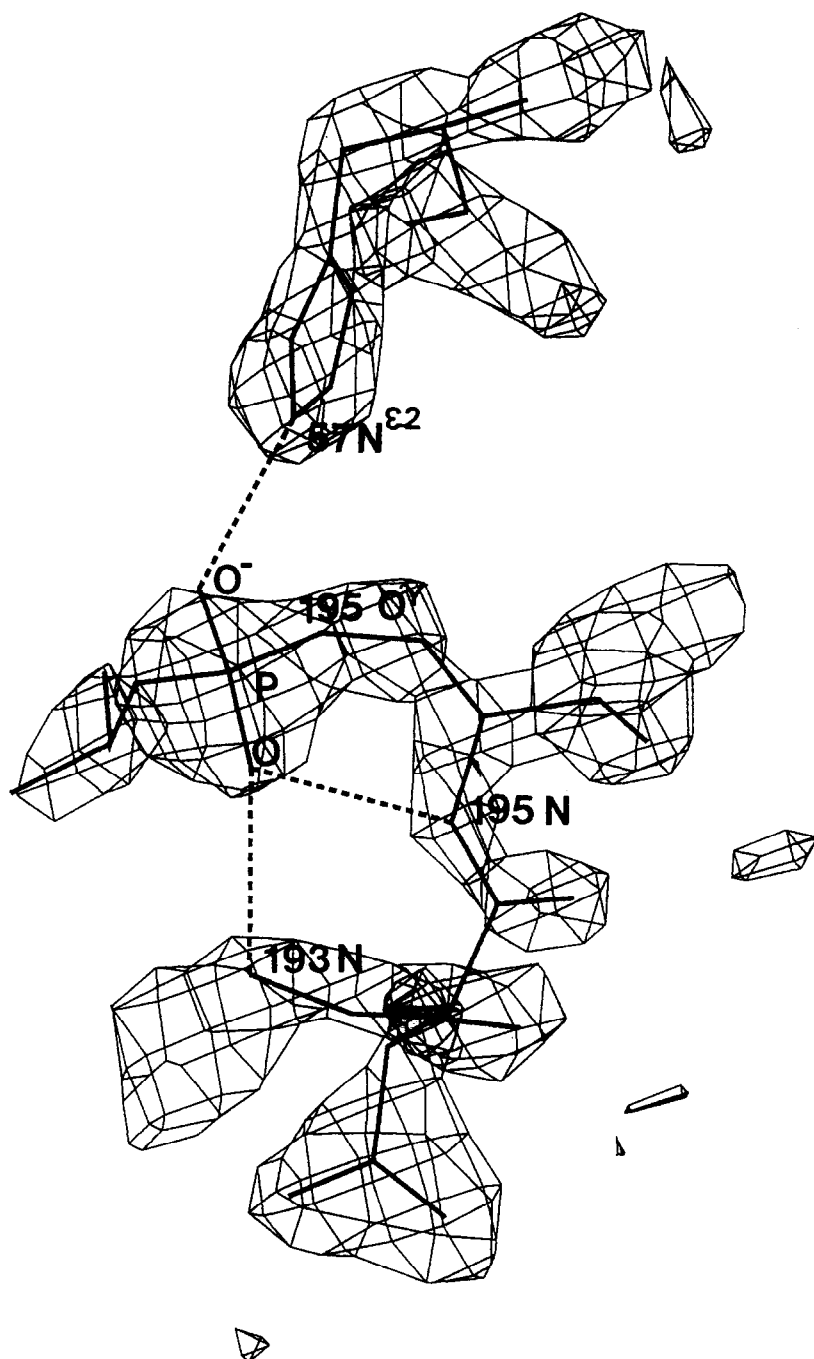


Figure 3. Electron density map, 6.0 to 2.1 Å resolution, with coefficients $(2F_o - F_c)$ drawn at 2σ , showing the bonding of the monoisopropylphosphoryl group in the active site of the "aged" conjugate, MIP- γ -Cht [$i\text{-C}_3\text{H}_7\text{O P(O)(O}^-\text{)-Cht}$]. Hydrogen bonds and charge-charge interactions are marked by broken lines.

(c) *Solvent structure*

During the iterative refinement process, water molecules were added by identifying peaks in the electron density map that were greater than 3σ in the $(F_o - F_c)$ map, greater than 1.5σ in the $(2F_o - F_c)$ map and within hydrogen-bonding distance of proton donors or acceptors. In the "non-aged" DEP- γ -Cht structure, three of these peaks, which still showed $(F_o - F_c) > 3\sigma$ after being assigned as waters, were changed to sulfate ions. In the MIP- γ -Cht structure, four sulfate ions were

included in the starting model, but two of the ions with the lowest occupancy showed no electron density in the refined structure and were deleted. At the final stages of the refinement of MIP- γ -Cht, the occupancy of one of the sulfate ions was fixed at 0.8 so that its isotropic temperature factors would be in the range of those of the rest of the solvent molecules.

The solvent water molecules in both conjugates were located independently. As expected, the structure of DEP- γ -Cht, which was determined at low-

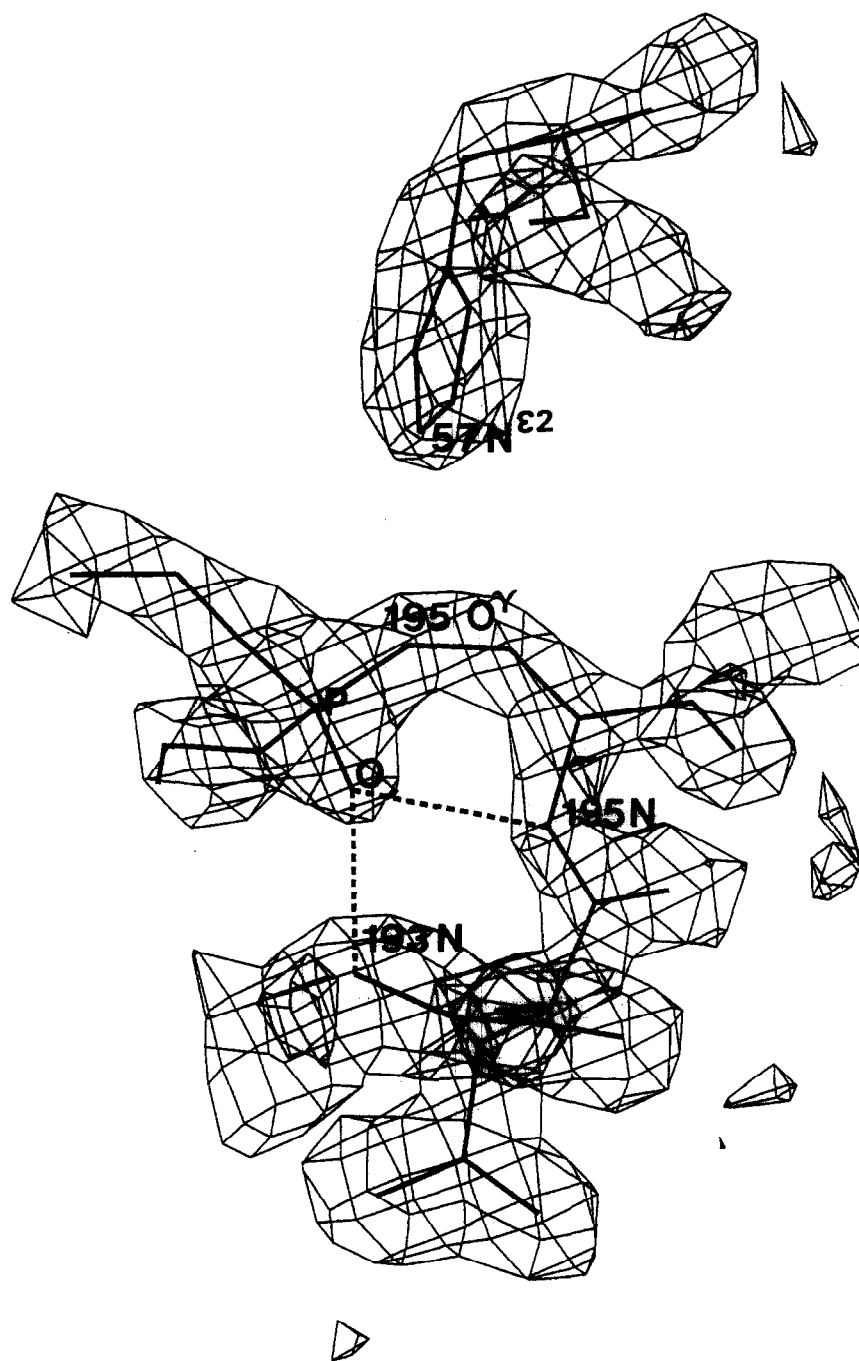


Figure 4. Electron density map, 6.0 to 1.9 Å resolution, with coefficients $(2F_o - F_c)$ drawn at 2σ , showing the bonding of the diethylphosphoryl group in the active site of the "non-aged" conjugate, DEP- γ -Cht $[(C_2H_5O)_2P(O)-Cht]$. Hydrogen bonds are marked by broken lines.

temperature and at higher resolution (1.9 Å), shows considerably more ordered water structure (278 molecules) than the room-temperature structure of MIP- γ -Cht determined at 2.1 Å (102 molecules). In the structure of DEP- γ -Cht, 227 water molecules were found when reflections with $F > 3\sigma$ were used in the refinement, and 51 additional solvent peaks were found when 25% more reflections were used by including the weaker data ($F > 0$). Comparison of the positions of the water molecules between the two structures, one of which was rotated so as to

form the best fit of its C^α positions to the other, shows that 81 out of 102 water molecules found in MIP- γ -Cht are equivalent, i.e. within a radius of 1 Å of water molecules found in DEP- γ -Cht. The distribution of these distances is shown in Figure 5. It is interesting to note that two of these equivalent pairs are found at the positions which are occupied in "native" γ -Cht by the aromatic ring of the scissile residue of the oligopeptide bound in the active site (Harel *et al.*, 1991) and both lie in the same plane as that of the aromatic side-chain.

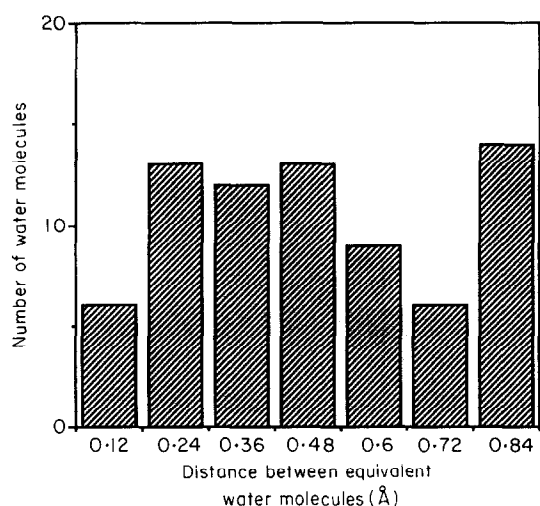


Figure 5. Histogram of distances between equivalent water molecules in the refined "aged" (MIP- γ -Cht) versus "non-aged" (DEP- γ -Cht) conjugates.

Although the refinement of MIP- γ -Cht started from the refined structure of native γ -Cht at 90 K and 1.6 Å resolution, with four sulfate ions (Harel *et al.*, 1991), the final density showed only two sulfate ions. In the DEP- γ -Cht structure, three sulfate ions were found. At the position of the fourth sulfate ion, which had the lowest occupancy in the native structure, the DEP- γ -Cht density was accounted for by a water molecule with an occupancy of 1.0 and a *B*-factor of 26.8 Å². Hence, as data to higher resolution are included, the solvent structure shows more detail.

4. Discussion

In order to explain the unusual resistance of "aged" OP conjugates of Cht to reactivation, as compared to similar "non-aged" conjugates, we compared the structures of representatives of both classes of conjugates at the atomic level. The structure of an "aged" conjugate, represented by MIP- γ -Cht, shows the formation of a strong interaction, 2.6 Å, between the negatively charged oxygen atom, which is produced by the loss of an isopropyl group subsequent to binding, and the protonated N²² of the imidazole ring of His57. This can explain the increased basicity (about 3 p*K*_a units) of a single titratable group, observed upon "aging" of DIP- γ -Cht and of similar conjugates of other serine proteases, by Adebodun & Jordan (1989), using ³¹P n.m.r. Thus in "non-aged" conjugates, His57 had a p*K*_a value near pH 7.4, whereas in the "aged" conjugates the p*K*_a shifted to much higher values, 9.7 to 11.4, depending upon the protease examined. An "aged" MEP conjugate of α -Cht displayed a similar effect (Y. Ashani *et al.*, unpublished results). The neutron diffraction studies of Kossiakoff & Spencer (1980, 1981) on MIP-trypsin also support the conclusion that a salt bridge is formed between the positively charged

imidazolium and the negatively charged, dealkylated, OP moiety.

Comparison of the refined structures of DEP- γ -Cht ("non-aged") and MIP- γ -Cht ("aged") shows virtually no conformational difference in the protein backbone overall (Fig. 2), in the active site (Fig. 6) or in the interaction Ile16-Asp194, between the two conjugates. Furthermore, the two structures superimpose very well on the protein backbone of both the native γ -Cht structure, with r.m.s. deviations of C α 0.16 Å for the "non-aged" and 0.28 Å for the "aged" superposition, and of the native α -Cht structure, with root-mean-square deviations of C α 0.42 Å for the "non-aged" and 0.44 Å for the "aged" superposition (when loop 75-79 is omitted). Ringe *et al.* (1986) reached a similar conclusion in comparing the overall three-dimensional structure of the stable acyl conjugate obtained by inhibition of γ -Cht with 3-benzyl-6-chloro-2-pyrone with that of the native enzyme. However, in the latter case, a reduction in distance of about 1 Å between Ser195 and His57 was reported upon binding of inhibitor.

The above conclusions are somewhat in conflict with the interpretation of earlier experiments in which pyrene-containing "aged" and "non-aged" OP conjugates of α -Cht were employed to demonstrate significant conformational differences between the "aged" and "non-aged" conjugates, using circular dichroism and other optical techniques (Steinberg *et al.*, 1989). If the pyrene group, as seems likely, is directed towards the hydrophobic pocket (Blow, 1976), there is nothing in our present X-ray data to indicate what might generate the spectroscopic differences observed. X-ray measurements of the corresponding pyrene-containing OP conjugates could settle this issue.

The refined structures of the "aged" and "non-aged" γ -Cht conjugates indicate that conformational changes of the polypeptide backbone, in or around the active site of the enzyme, do not lie at the basis of the unusual resistance of the "aged" conjugates to reactivation by nucleophiles. In addition, it was already pointed out that the negative charge as an electrostatic barrier cannot alone be responsible for the unusual resistance to reactivation (Amitai *et al.*, 1982). The most likely explanation for the observed resistance to reactivation is, therefore, that a unique interaction between the P-O⁻ group and the positively charged imidazole of His57 imposes a severe restraint on the reactivation process. The observation that denaturation of an "aged" OP-Cht conjugate permits displacement of the phosphoryl moiety (Y. Ashani *et al.*, unpublished results) supports this contention.

An analogous situation to the above was encountered by Ringe *et al.* (1985) in their X-ray and n.m.r. studies on the stable acyl-Cht conjugate produced by interaction of 3-benzyl-6-chloro-2-pyrone with γ -Cht. The stability of the conjugate which they observed was explained by them as being due to an ionic interaction (3.4 Å) of the protonated form of His57 with a free carboxylate group on the inhibitor

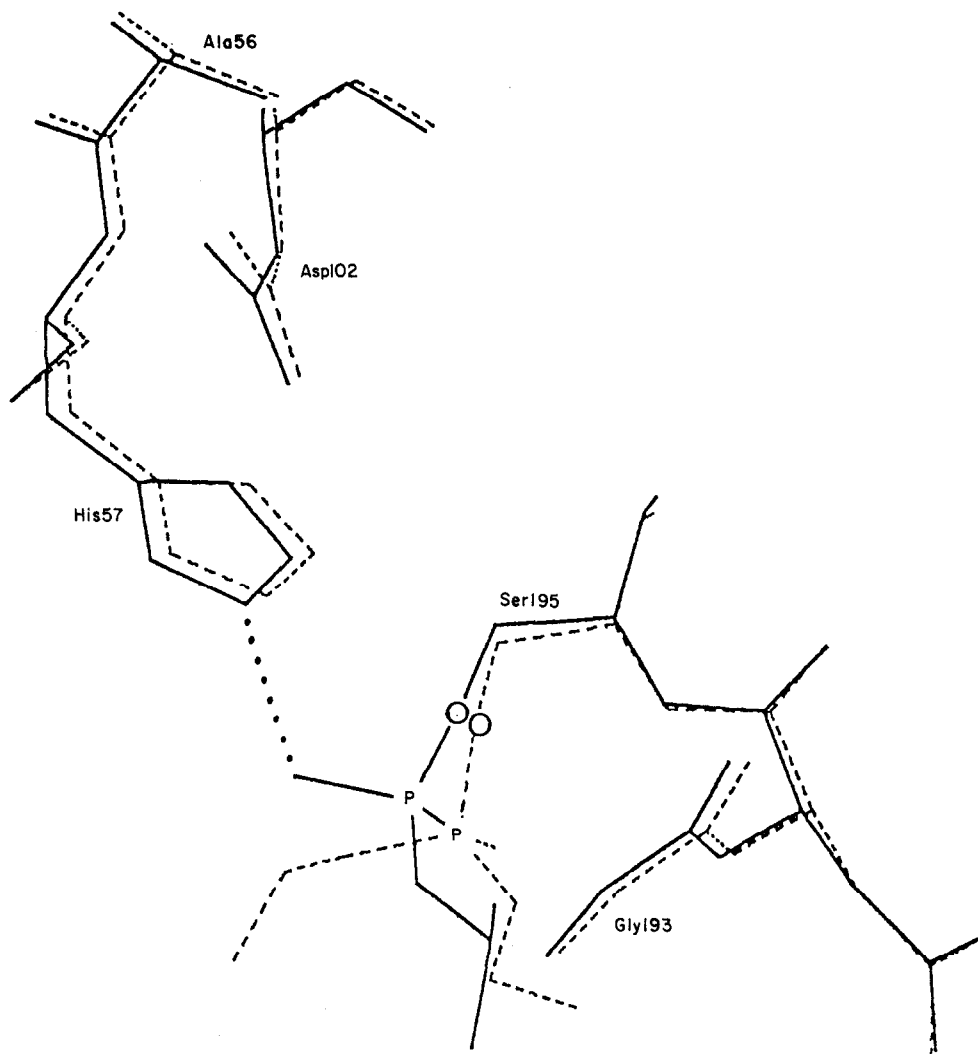


Figure 6. Overlap of the active site residues and the bound OPs of "aged" MIP- γ -Cht (continuous line) and "non-aged" DEP- γ -Cht (broken line). The charge-charge interaction between O^- of MIP and His57 $N^{\epsilon 2}$ is shown as a dotted line.

moiety of the conjugate. In this conjugate, slow deacylation occurs, and was explained by a model in which the carboxylate moiety is able, occasionally, to rotate away from the imidazole ring thus lowering the latter's protonation state and restoring the general base function (Ringe *et al.*, 1986).

Since conformational changes in the protein do not lie at the basis of the resistance to reactivation, nor does the electrostatic factor alone, a molecular basis for the phenomenon must be sought elsewhere. It may be envisioned that the stereochemical requirements for a displacement reaction to occur at the phosphorus atom (Hall & Inch, 1980) cannot be fulfilled in the "aged" conjugate due to the constraints imposed by the salt bridge. For example, if a penta-co-ordinated intermediate is formed during the reactivation of an OP-Cht in the presence of a nucleophile, it is possible that the interaction between the $P-O^-$ group and the protonated $N^{\epsilon 2}$ of His57 will prevent the ligands attached to the phosphorus atom of the "aged" conjugate from assuming a geometry (e.g. equatorial *versus*

apical) favorable for the enzyme to depart as a leaving group, i.e. to be reactivated.

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