# SPECIFIC AND NON-SPECIFIC BINDING OF AN ORGANOPHOSPHATE SPIN LABEL TO SOME SERINE-ESTERASES

A.J. HOFF*

*Laboratory of Physiological Chemistry, University of Leiden, The Netherlands*

R.A. OOSTERBAAN

*Medical Biological Laboratory TNO, Rijswijk Z.H., The Netherlands*

and

R. DEEN

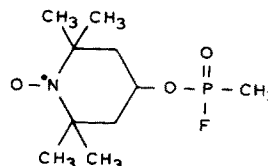*Central Laboratory TNO, Delft, The Netherlands*

## 1. Introduction

Spin labelling has become an important tool in probing the structure of biological materials. Recently organophosphate spin labels have been described which were reported to react specifically with the serine residue in the active site of serine-esterases [1, 2]. This offers the possibility of comparing the spatial structure surrounding the active serine in these enzymes. In this paper the results are given of an ESR study on a bacterial atropinesterase [3], on subtilisin and on chymotrypsin, which were reacted with the organophosphate spin label 1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl methylphosphonofluoridate. It was found that this spin label to some extent also bound non-specifically to subtilisin and chymotrypsin, making a detailed comparison of the ESR spectra of these enzymes with that of atropinesterase difficult. This non-specific binding could be largely suppressed by modifying the experimental conditions. The spectra then found differed only slightly for the three enzymes. They were typical for almost totally immobilized spin label.

## 2. Materials and methods

Subtilisin was purchased from NOVO Industri A/S, chymotrypsin from Nutritional Biochemical Corporation. Atropinesterase was isolated from *Pseudomonas putida* and purified as described elsewhere [3]. All enzymes were pure as judged from polyacrylamide gel-electrophoresis SDS and urea.

The phosphonate spin label was prepared according to Morrisett, Broomfield and Hackley [1]. The starting material 1-oxyl-2,2,6,6-tetramethylpiperidinol-4 was synthesized according to Brière, Lemaire and Rassat [4]. The product was digested with $CCl_4$ and further purified by recrystallization, giving orange crystals (mp 69°, correct elementary analysis and IR spectrum). The phosphonate



was purified by molecular distillation ( el. analysis P 12.68, F 7.05; calc. 12.28 and 7.53 resp.; correct IR spectrum). The ESR signal consisted of the usual three lines with a partly resolved proton hyperfine structure.

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\* Present address: Medical Biological Laboratory TNO, 139 Lange Kleiweg, Rijswijk Z.H., The Netherlands.
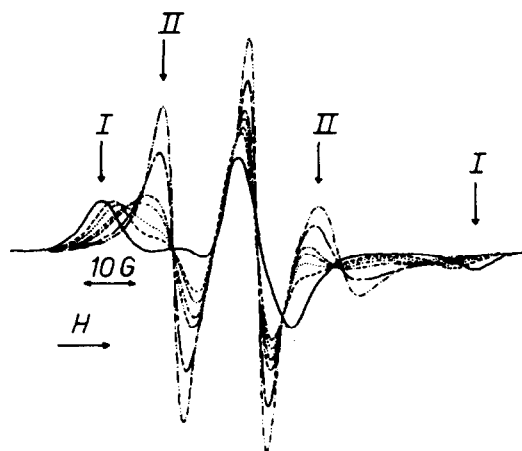
Fig. 1. ESR spectra of 0.16 mM 1-oxyl-2,2,6,6-tetramethyl-
piperidinol-4 in a mixture of 53% glycerol in water at different
temperatures. Gain 100, modulation 2G, power 10 mW. Arrows
labelled I and II indicate features characteristic of strongly and
weakly immobilized spin label respectively. (– ·· –) $0°$, $\tau_c$ =
4.5 nsec;(-- - —)–4°, 6 nsec; (-- ··--) –10°, 9 nsec; (-· -··-) –12°,
12 nsec; (. . . . ) –14°, 30 nsec; (-----) –20°, 60 nsec; (——)
–74°, 120 nsec.

Enzyme solutions of around 0.1 mM in 10 ml 0.02
M tris-HCl buffer of pH 8.3 were treated at 25° with
a solution of 0.1 M spin label in isopropanol. To re-
move free label the mixture was extensively dialysed
at 4° against the tris buffer with 0.1 M KCl added and
then concentrated about 10 times by ultradialysis. In
some experiments the excess free label was removed
by gel-filtration at 4° on Sephadex G50 in the high salt
buffer. As a control the enzymes were completely in-
hibited at 25° by excess DFP (diisopropyl phosphoro-
fluoridate), dialysed in the cold against the high salt
buffer, subsequently dialysed against the tris buffer
without KCl and then treated with spin label as above.
Between successive steps preparations were kept at
0°, samples were stored at –18°. After each step and
after the ESR measurements the preparations were
tested for recovery of enzyme activity by spontaneous
dephosphorylation. This proved not to occur under
the experimental conditions. Enzyme activity was
determined by conventional pH-stat method using
$N$-acetyl-L-tyrosine ethyl-ester and (–)-atropine as
substrates.

ESR spectra of the enzyme preparations were
measured at 0° with a JEOLCO ME-IX spectrometer.
Thin glass capillaries containing the samples were cen-

tred along the axis of the cylindrical cavity. Reference
spectra were obtained by recording the spectrum of
a sample of the oxyltetramethylpiperidinol dissolved
in a glycerol–water mixture over a range of tempera-
tures (fig. 1). The shape of the ESR spectrum of the
spin label, which reflects its freedom of motion, was
characterized by the correlation time $\tau_c$. The values of
$\tau_c$ were based on the estimation of Hsia and Piette [5]
for the same radical in glycerol–water mixtures. Al-
though the absolute values of $\tau_c$ are not reliable, the
method is adequate for qualitative comparison. Since
the reference spectra were measured on one and the
same sample, they could be used to relate the height
of lines at various correlation times with the amplitudes
of the completely isotropic spectrum. In this way
the concentration ratios of isotropic and anisotropic
components in mixed spectra were determined.

## 3. Results and discussion

The spin label proved a very effective inhibitor for
subtilisin, chymotrypsin and atropinesterase, the $k_I$
being approximately $3 \times 10^4$, $1 \times 10^5$ and
$2 \times 10^4$ $M^{-1}$ $min^{-1}$ respectively.

The spectrum of spin-labelled atropinesterase is
given in fig. 2a. It has the shape of the anisotropic,
broadened spectrum typical for strongly immobilized
label. Superimposed is a minor component, charac-
teristic of weakly immobilized label. This minor com-
ponent amounts to less than one percent of the total
spin concentration. Since the enzyme is completely
inhibited the anisotropic spectrum, designated type I,
must be due to spin label which is specifically bound
to the active serine. The mobility of the label depends
on the tertiary structure of the enzyme, as is demon-
strated by the ESR spectrum of the same sample,
after denaturation with 4.5 M urea (fig. 2b), showing
an increased mobility as expected.

The ESR spectra of completely inhibited subtilisin
and chymotrypsin differ markedly from that of
labelled atropinesterase (fig. 2c and d). Here the iso-
tropic spectrum (type II), typical for weakly immo-
bilized label, is much more prominent. Consequently
in both spectra the low field region of the anisotropic
component is distorted, which hampers detailed com-
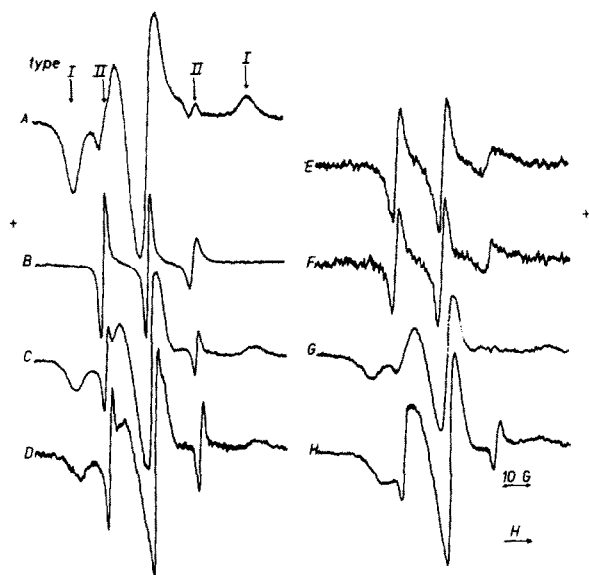parison of this component with the atropinesterase
spectrum.

Fig. 2. ESR spectra of spin-labelled atropinesterase, subtilisin
and chymotrypsin.

a) 0.5 mM atropinesterase labelled during 60 min with 2.0 mM
   spin label at 25° to 99.0% inhibition
b) atropinesterase from a) denatured with 4.5 M urea
c) 0.18 mM subtilisin labelled for 40 min with 0.5 mM spin
   label at 25° to 99.5% inhibition
d) 0.4 mM chymotrypsin labelled for 30 min with 2.0 mM
   spin label at 22° to 99.3% inhibition
e) and f) 0.07 mM subtilisin and 0.1 mM chymotrypsin re-
   spectively, inhibited for 15 min with 1.0 mM DFP at 22°
   to 99.7% and 99.4% inhibition respectively, dialyzed first
   against 0.02 M tris-HCl buffer pH 8.3 and then against the
   same buffer with 0.1 M KCl added, then treated for 15 min
   with 0.5 mM spin label at 22°. Gel-filtration on Sephadex
   G 50 at 0° in the high salt buffer, and then concentrated
   10 times by ultradialysis
g) and h) 0.07 mM subtilisin and 0.1 mM chymotrypsin
   labelled with 0.04 and 0.05 mM spin label to 57% and
   65% inhibition respectively, then treated for 30 min with
   1.5 mM DFP at 22° to 99.5% and 99.3% inhibition respec-
   tively. Gel-filtration and concentration as above
   Spectrometer data: frequency 9.26 GHz; field sweep ± 50 G.
   mod. width 2 G, except b and c: 1G; resp. 0.3 or 1 sec,
   sweep time 5 or 10 min; power 10 mW; gain for a–h:
   360, 560, 220, 560, 790, 1000, 360 and 500 respectively

The high intensity of the type II signal in subtilisin
and chymotrypsin is rather puzzling. Organophosphate
inhibitors usually bind exclusively to the active serine
under the conditions of our experiment [6]. Published
spectra of esterases labelled with the same spin label as
used in this work do not show the complex pattern

which we found [1]. The type II signal can not be due
to spin label released by reactivated enzyme since be-
fore and after the ESR experiments all enzymes were
completely inhibited ($>99\%$). Moreover, a similar
signal was present in the controls consisting of enzymes
which were completely inhibited with DFP prior to
spin label treatment, as shown in fig. 2 e and f. The
correlation time of this signal is 0.2 nsec. This indicates
that the mobility of the nitroxide group of the spin
label is appreciable but not yet as high as that of free
label.

Conceivably the type II signal could be due to
1) absorbed, but not covalently bonded spin label,
2) spin label bonded to products of autolysis in the
   case of subtilisin and chymotrypsin,
3) denaturation of protein after spin labelling,
4) different mobility of the bonded R and S stereo-
   isomers of the spin label, which contains an
   asymmetric phosphorus atom,
5) reaction of the spin label with non-specific sites,
   resulting in a weaker immobilization.

In the following the foregoing possibilities are dis-
cussed.

The presence of absorbed spin label is unlikely,
because extensive dialysis against high salt buffer led
to essentially unchanged spectra.

Since autolysis may occur during the period that
the proteolytic activity of subtilisin and chymotrypsin
is not yet inhibited completely, the formation of
labelled protein fragments cannot be excluded a priori.
Therefore the experiments were repeated with pre-
parations that had been subjected to gel-filtration on
Sephadex G 50 after the inhibition. All proteins were
eluted as a single peak as measured by the absorbancy
of the fractions at 280 nm. The fractions were pooled
in three roughly equal portions, corresponding to the
front, centre and last section of the peak, and con-
centrated by ultradialysis. For subtilisin, which does
not contain S–S bridges, labelled fragments would be
expected to be eluted after the protein peak if auto-
lysis had occurred, whereas for both proteases a dif-
ferent composition of the first and last section of the
elution peak might be expected. However, all labelled
enzymes showed identical spectra for all three sections
of the elution peak, which did not differ from the
ones recorded for samples prepared without gel-filtra-
tion. Furthermore the intensity of the type II signal
did not increase when the labelled enzymes were in-

cubated for 15 minutes with equal amounts of an active proteolytic enzyme. These results exclude labelled products of autolysis as the source of the type II signal.

In enzymes pretreated with excess DFP, the active site is no longer available for labelling. Accordingly, in the spectra of these preparations the type I signal was not present. Yet, as shown in fig. 2 e and f, these samples exhibit a type II spectrum nearly as intense as the samples that were not treated with DFP (fig. 2 c and d). Apparently the type II signal is not due to denaturation of the protein after labelling at the active site.

In some experiments the enzymes were inhibited completely with approximately stoichiometric amounts of spin label. Assuming a different mode of binding for the R and S isomers of the spin label, one would expect nearly equal amounts of weakly and strongly immobilized spin label. However, the concentration of spin label giving rise to the type II signal was about one tenth of that of the type I signal, indicating that the presence of the type II spectrum is not due to one of the R and S forms of the spin label.

If the type II signal is due to non-specific binding sites, one would expect at least one such site per enzyme molecule. However, from the spectra of fig. 2 c and d it was calculated that the concentration of type II sites is only 7.0% and 15.2% of the concentration of the type I sites for subtilisin and chymotrypsin respectively. Prolonged incubation of DFP inhibited subtilisin with excess spin label did not enhance the low concentration of weakly immobilized label. From the data and the known enzyme concentration it was calculated that only about 5 percent of inhibited enzyme molecules possess an accessible non-specific site, which apparently does not react with DFP. In ordinary enzymological work this small fraction will usually remain undetected, but in ESR experiments with the sensitive spin label technique it makes interpretation of spectra hazardous unless precautions are taken to reduce its effect.

It was found that the reaction of spin label with the non-specific sites could be suppressed to a large extent by incubating subtilisin and chymotrypsin with a less than stoichiometric amount of spin label. From the spectra obtained (fig. 2 g and h) and from the spectrum of atropinesterase (fig. 2a) the correlation time of the strongly immobilized spin label was

determined. For atropinesterase, subtilisin and chymotrypsin it amounted to 37, 44 and 15 nsec respectively. These values are approximately equal to the tumbling time of the protein molecules as estimated from the Einstein-Stokes relation (about 20 nsec), indicating that spin label attached to the active site is almost completely immobilized relative to the protein molecule. This result is in contrast with the data of Morrisett et al. [1], who investigated the binding of the same compound to acetylcholinesterase and to chymotrypsin at pH 4; in both cases the spin label was only partly immobilized relative to the protein.

The lower value of the correlation time for chymotrypsin with respect to those for the other enzymes cannot be explained on the basis of their respective molecular weights, which are only slightly different. It follows that the $-NO^\cdot$ group of the spin label has an increased motional freedom at the active site of chymotrypsin. This site is reported to form a pocket or slit whose dimensions are about 11 Å by 6 Å by 4 Å [7]; presumably the bulky tetramethylpiperidinyl group (diameter about 10 Å) cannot be accommodated fully in the rather narrow slit, but will protrude partially into the solvent. Apparently the active site of subtilisin and that of atropinesterase is wider and thus the spin label is more fully immobilized in these enzymes. Whether the slight difference in correlation times between subtilisin and atropinesterase is significant will require further investigation.

## Acknowledgements

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