Biochimica et Biophysica Acta, 708 (1982) 266–271
Elsevier Biomedical Press

BBA 31363

^{1}H-NMR SPECTROSCOPIC STUDY ON THE BINDING OF ALCOHOLS TO SOYBEAN LIPOXYGENASE-1

STEVEN SLAPPENDEL [a], ROLAND AASA [a], KARL-ERIK FALK [a], BO G. MALMSTRÖM [a], TORE VÄNNGÅRD [a], GERRIT A. VELDINK [b] and JOHANNES F.G. VLIEGENTHART [b]

[a] *Chalmers Institute of Technology and University of Göteborg, Department of Biochemistry and Biophysics, 412 96 Göteborg (Sweden) and* [b] *State University of Utrecht, Department of Bio-organic Chemistry, Croesestraat 79, 3522 AD Utrecht (The Netherlands)*

(Received June 4th, 1982)

Key words: Lipoxygenase; ^{1}H-NMR; Alcohol binding; Line broadening ; (Soybean)

The line-shape of the EPR signal around *g* 6 of yellow lipoxygenase-1, obtained upon addition of 1 molar equivalent of 13-L$_S$-hydroperoxy-9-*cis*,11-*trans*-octadecadienoic acid to the native enzyme (linoleate:oxygen oxidoreductase, EC 1.13.11.12), is strongly affected by alcohols. NMR spectra of solutions of alcohols to which lipoxygenase has been added show a line-broadening of the proton resonances which is due to proton relaxation enhancement from magnetic interaction between iron and protons. This can be taken as direct evidence for binding of alcohols in the vicinity of iron. For unbranched alcohols the line-broadening gradually increases, going from the methyl protons to the protons on carbon atom 1, indicating that the latter are closer to iron. Titrations of yellow lipoxygenase with ethanol, 1-butanol and 1-hexanol reveal that the affinity of the alcohols increases with longer carbon chain length; their binding constants were found to be 260, 30 and approx. 3 mM, respectively. The distances between protons of bound alcohol and iron were calculated with the Solomon-Bloembergen equation, leading to values of approx. 6 Å for the distance between iron and the methyl protons. A hydrophobic binding of the alcohols to the enzyme is proposed in line with the mode of binding of the natural substrates, polyunsaturated fatty acids.

Introduction

Lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) is a non-heme iron dioxygenase which catalyzes the peroxidation of polyunsaturated fatty acids containing a 1,4-*cis,cis*-pentadiene structure [1]. The native, colourless and EPR-silent enzyme can be converted into a yellow enzyme form by addition of 1 molar equiv. of 13-L-HPOD, the main product of the reaction of the enzyme with linoleic acid at pH 9.0 under aerobic conditions. Yellow lipoxygenase shows a complex EPR signal around *g* 6 [2–4] arising from at least three high-spin Fe(III) species [5] differing in their degree of rhombicity [6].

The number and relative amounts of the species and thus the line-shape of the EPR spectrum around *g* 6 is affected by pH, ionic strength, potassium cyanide and alcohols [6]. In particular, alcohols induce a shift to a nearly axial type of EPR spectrum [6]. For example, only 8 mM ethanol (0.05% v/v) is required to get a complete shift. It is known [7] that much higher concentrations of alcohols (25% v/v) cause changes in the EPR spectra which are, however, attributed to changes of the structure of the frozen solution. Therefore, in the case of lipoxygenase a more specific interaction with the alcohols is likely. Mitsuda et al. [8]

Abbreviation 13-L-HPOD, 13-L$_S$-hydroxyperoxy-9-*cis*,11-*trans*-octadecadienoic acid.

0167-4838/82/0000-0000/$02.75

have described the inhibition of lipoxygenase by saturated monovalent alcohols which probably bind to the enzyme at a hydrophobic region serving also as a binding site for the substrate.

An EPR study [6] has revealed that alcohols have an effect on the conformation of the active site, but for a characterization of possible binding of alcohols this method is inadequate. A ^{1}H-NMR study, described in this article, has provided information on both the way of binding and the binding constants of alcohols to lipoxygenase-1.

Materials and Methods

Lipoxygenase-1 was isolated from soybeans according to the method of Finazzi-Agrò et al. [9] with modifications as reported by Galpin et al. [10]. The specific activity of the enzyme was 3.92 μkat · mg^{-1}, corresponding to 235 μmol O_2 · min^{-1} · mg^{-1}. The iron content was 0.97 mol per mol enzyme and the amounts of Mn and Cu were found to be 0.07 and 0.006 mol per mol enzyme, respectively [5].

Linoleic acid (purity > 99%) was obtained from Lipid Supplies (St. Andrews University, St. Andrews, U.K.). 13-L-HPOD was prepared by aerobic incubation of linoleic acid with soybean lipoxygenase-1 at pH 9.0 [11] and purified by HPLC according to the method of Verhagen et al. [12]. The other chemicals used were of reagent grade.

NMR spectra were recorded on a Bruker 270 MHz spectrometer operating in the Fourier transform mode. The temperature was 297 K. The enzyme and alcohol solutions were prepared with 0.1 M boric acid in ^2H_2O brought to pH 9.0 with NaOH. No correction was made for the deuterium effect. The room temperature EPR spectrum at 9.5 GHz was recorded on a Varian E-9 spectrometer using a flat aqueous solution cell.

Results

The NMR spectra of a solution of 1-butanol in 0.1 M borate buffer, pH 9.0, before and after addition of yellow enzyme in a molar ratio 1:855 (enzyme:alcohol) are shown in Fig. 1. The presence of the enzyme leads to line-broadening of the proton resonances from the alcohol, which is due

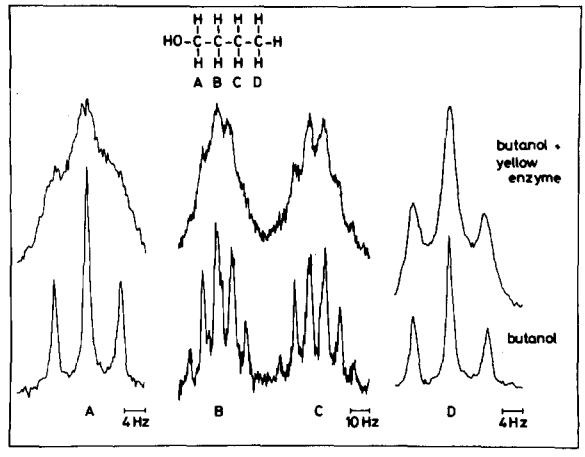

Fig. 1. NMR spectra of 1-butanol showing the paramagnetic effect of iron in yellow lipoxygenase-1 on the proton resonances of 1-butanol. Lower spectra, 8 mM 1-butanol; upper spectra, yellow lipoxygenase (26 μM) and 1-butanol (22.8 mM). Buffer, 0.1 M borate/^2H_2O, pH 9.0. Temperature, 297 K.

to paramagnetic relaxation enhancement [13], this implying that an interaction exists between iron and the alcohol. The line-broadening is smaller for the methyl protons than for the protons bound to carbon atom 1. Table I gives the line-broadening effects of yellow enzyme on the methyl protons

TABLE I

LINEWIDTH INCREMENTS OF METHYL PROTONS AND PROTONS BOUND TO CARBON ATOM 1 OF VARIOUS ALCOHOLS OBSERVED UPON ADDITION OF YELLOW Fe(III) LIPOXYGENASE

The alcohol concentration was 8 mM for the ratio 1:300 and 24 mM in the other cases. The error in the increments of up to 5.0 Hz is approx. ±0.2 Hz.

Alcohol	Molar ratio [a]	Observed line-broadening (Hz)	
		Methyl protons	Protons bound to C_1
Ethanol	1: 300	1.5	2.0
1-Butanol	1: 300	5.0	≈11
t-Butanol	1: 300	0.4	
1-Hexanol	1: 300	≈8	≈21
	1:2100	1.6	5.0
	1:4300	0.8	2.2
	1:8500	0.4	1.2

[a] Molar ratio lipoxygenase:alcohol.

and the protons bound to carbon atom 1 of various alcohols.

The methyl proton resonances of ethanol, 1-butanol and 1-hexanol, recorded after the addition of yellow enzyme in a molar ratio of 1:300 (enzyme:alcohol) are shown in Fig. 2. The linewidths clearly increase with longer carbon chain length.

Titration curves of ethanol and butanol with yellow enzyme are shown in Fig. 3. The results are presented in a similar way to those for NMR-shift experiments [14,15].

For a system in rapid equilibrium (A + E ⇆ AE) one can derive [15]

$$A_0 = E_0 \Delta_b \frac{1}{\Delta \nu} - K_A - E_o \qquad (1)$$

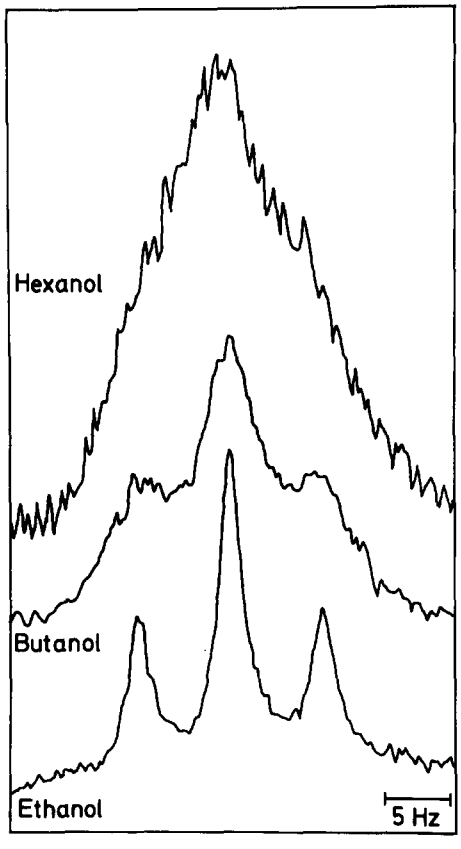

Fig. 2. Methyl proton resonances of ethanol, 1-butanol and 1-hexanol recorded after addition of yellow lipoxygenase. Concentrations: lipoxygenase, 0.27 mM; alcohol, 7.59 mM. Temperature, 297 K. Without enzyme the linewidth of the methyl resonances was approx. 1 Hz.

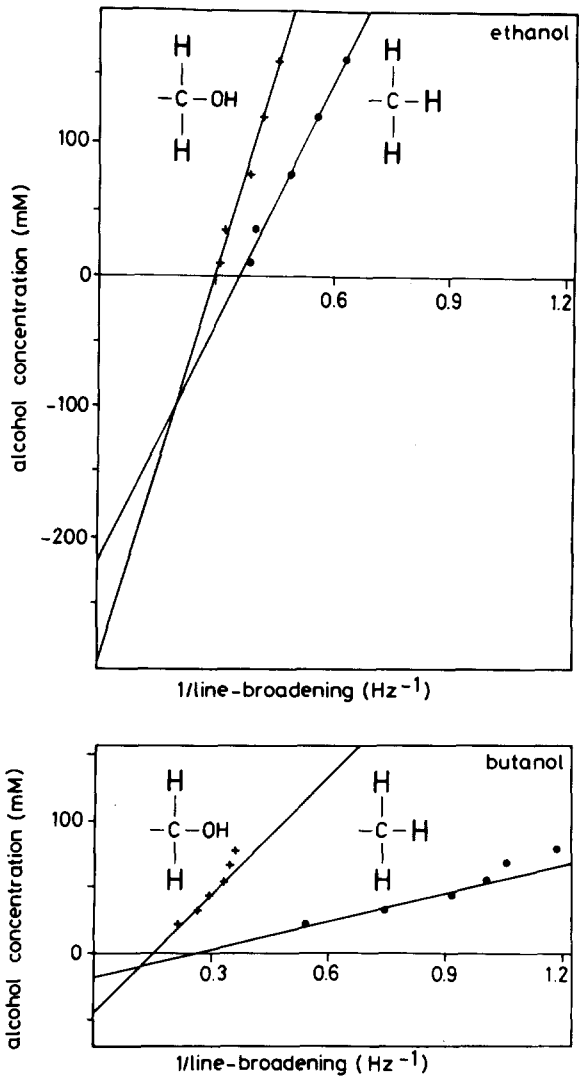

Fig. 3. Titration of yellow lipoxygenase with ethanol and 1-butanol. Upper figure: a yellow enzyme solution (50 μM in 0.1 M borate buffer/^2H_2O, pH 9.0) was titrated with ethanol (10.6 M in borate buffer/^2H_2O, pH 9.0). Lower figure: a yellow enzyme solution (27 μM) was titrated with pure 1-butanol (10.9 M). The line-broadening was determined from comparisons with simulated spectra. For 1-butanol only the experimental points at low concentration were used for the least-square fitting procedure.

where A_0 and E_0 are the total alcohol and enzyme concentrations, respectively, Δ_b is the linewidth of the alcohol-enzyme complex and $\Delta\nu$ is the observed line-broadening. Eqn. 1 shows that a larger linewidth corresponds to a smaller K_A, i.e., higher

affinity for binding. From a plot of A_0 against $1/\Delta\nu$, K_A and Δ_b can be calculated from the intercept and slope, respectively. In this model it is assumed that all alcohol molecules bind at the same site. In the case of more than one binding site the same equation holds and describes an average situation, provided that at a given moment one alcohol molecule at most is bound per enzyme molecule.

For ethanol (Fig. 3A) a linear relation is obtained, but for 1-butanol (Fig.3B) a deviation from linearity is found. This might indicate that more than one molecule of 1-butanol can be bound per enzyme molecule. This phenomenon has also been reported by Mitsuda et al. [8]. 1-Hexanol could only be used for a limited number of measurements because of its low solubility. Furthermore, the large width of the methyl proton resonances of hexanol lowers the accuracy of the determination of the line-broadening.

From the binding model described above it might be expected that identical intercepts are to be found for the protons at different carbon atoms in one particular alcohol. However, a least-square fitting of the data made separately for the methyl protons and for the protons bound to carbon atom 1 leads to different intercepts (Fig. 3). Normal experimental error or inadequate description of the binding by the model used (intramolecular rotations have not been taken into account) could be the reason for this inconsistency. The mean values of the binding constants, K_A, are given in Table II. The data demonstrate that the affinity of the alcohols for binding to yellow lipoxygenase increases with their carbon chain length.

The linewidth of bound alcohol, Δ_b (Table II), which is related to the transverse relaxation time by the equation $1/T_{2M} = \pi\Delta_b$, can be employed for an estimate of the distances (r) of the protons to the paramagnetic center, i.e., Fe(III) ($S = 5/2$, $g = 2$) for yellow lipoxygenase.

Although limitations exist for the application of the Solomon-Bloembergen equation [16,17], this equation (Eqn. 2) [13] can be used in the region of fast exchange.

$$\pi\Delta_b = \frac{1}{T_{2M}} = \frac{1}{15}\,\frac{\gamma^2 g^2 S(S+1)\beta^2}{r^6} \times \left[4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{13\tau_c}{1+\omega_S^2\tau_c^2}\right] \qquad (2)$$

Fast exchange of bound hexanol with the bulk hexanol is probable because the calculated values of the linewidth of bound hexanol, Δ_b, are constant for different concentrations at high molar ratios (Tables I and II). The same is true for the other alcohols because of the observed linear relationship between the alcohol concentration and the reciprocal line-broadening ($1/\Delta\nu$) (Fig. 3).

For an estimation of the correlation time τ_c we can use

$$\frac{1}{\tau_c} = \frac{1}{\tau_R} + \frac{1}{\tau_S} + \frac{1}{\tau_M}$$

If the alcohols are bound to lipoxygenase, their rotational correlation time τ_R can be taken as that of the whole protein molecule and can be esti-

TABLE II

QUANTITATIVE RESULTS OF THE TITRATIONS OF YELLOW ENZYME WITH VARIOUS ALCOHOLS AND CALCULATED DISTANCES BETWEEN IRON AND METHYL PROTONS AND PROTONS BOUND TO CARBON ATOM 1, RESPECTIVELY

Alcohol	K_A (mM)	Δ_b (kHz)		Distance to iron (Å)	
		CH_3-	-CH_2OH	CH_3-	-CH_2OH
Ethanol	260 [a]	11.9	20.1	5.1	4.7
1-Butanol	30 [a]	1.6	4.7	7.2	6.0
1-Hexanol [b]	≈3	3.4	10.1	6.3	5.3

[a] Mean values obtained from Fig. 6.

[b] Data derived from Table I. For the calculation of Δ_b, K_A has been neglected.

mated by the Stokes-Einstein equation $\tau_R = 4\pi r^3/3kT$. With $r = 4.5$ nm [18], $\eta = 1.0$ cp, τ_R is calculated to be $0.9 \cdot 10^{-7}$ s at 297 K. The exchange lifetime τ_M is often found to be in the μs range [19–21] and, hence, does not contribute to the correlation time. Preliminary T_1 measurements on samples prepared as described in Fig. 1 give $T_{1M}/T_{2M} = 30$ for all protons in 1-butanol from which τ_c is estimated to be $4 \cdot 10^{-9}$ s [21], therefore defined by τ_S. This value is consistent with $\tau_S > 2 \cdot 10^{-10}$ s as obtained from the linewidth of the room temperature EPR spectrum (20 mT) (cf. Ref. 22). For our estimates we have used $\tau_c = 1 \cdot 10^{-9}$ s, which gives from Eqn. 2 the relation

$$r^6 = \frac{2.2}{\Delta_b} \cdot 10^8$$

where r is the distance (in Å) between the proton and the iron atom.

The results for ethanol, 1-butanol and 1-hexanol are presented in Table II. Under the assumptions implicated in Eqn. 2 [16,17], the absolute values of the distances may be off by 2 Å but for all alcohols used the distance between the protons bound to carbon atom 1 and the iron atom is shorter than the distance between the iron atom and the methyl protons.

In order to study the mode of binding of the alcohols we have added KCN to an ethanol/yellow enzyme solution (ethanol 8 mM, enzyme 27 μM, KCN 8 or 80 mM). KCN also induces a shift to a more axial type of EPR spectrum [6] and has an affinity for binding to the enzyme similar to that of 1-butanol (unpublished results). No change in linewidth of the proton resonances of ethanol was observed.

Discussion

In a study concerning the nature and relative amounts of the high-spin Fe(III) which build up the complex g 6 signal in the EPR spectrum of yellow lipoxygenase a large effect of alcohols on the relative amounts of high-spin Fe(III) species has been reported [6]. The interaction of alcohols with lipoxygenase has now been studied in more detail with ^{1}H-NMR spectroscopy. The line-broadening observed upon addition of yellow lipoxygenase to 1-butanol (Fig. 1) is caused by proton relaxation enhancement from the magnetic interaction between iron and protons. This means that alcohol is bound in the environment of iron [13]. The binding of alcohol to other places of the protein chain of the enzyme can be excluded, because the magnitude of the linewidth of bound alcohol (Table II) is at least 100-times larger than the linewidth of various resonances in the proton NMR spectrum of lipoxygenase [23].

The increase in affinity of the alcohols for binding to yellow lipoxygenase with increasing carbon chain length is clear from the binding constants given in Table II. However, for *t*-butanol only a small line-broadening is observed compared to the other alcohols (Table I), corresponding to a much lower affinity for binding (Eqn. 1), probably caused by steric hinderance. This is consistent with the EPR results [6] also showing a low affinity of *t*-butanol. Furthermore, a shift to a more rhombic type of EPR spectrum of yellow lipoxygenase is observed upon addition of *t*-butanol, indicating a different mode of binding.

In principle the alcohol can bind to the enzyme via the hydroxyl group or via the alkyl part of the molecule. From the competition experiment with cyanide a binding of the alcohols via the hydroxyl group is found to be improbable because no change in linewidth is observed upon addition of cyanide to an ethanol/yellow enzyme solution. Binding via the alkyl part of the molecule implies a hydrophobic interaction between the alkyl part of the alcohol and the enzyme. Also for the binding of the substrate fatty acid, a hydrophobic interaction with the enzyme is more likely than a binding via the carboxyl group, because from studies on the substrate specificity of lipoxygenase [24] it is clear that the structure of the hydrophobic part is critical to the specificity of the enzyme. The *n*-6 fatty acids are the best substrates. From a model of the molecule it can be deduced that with the double bonds in the *n*-6 and *n*-9 positions the distance between the methyl protons and the methylene protons at *n*-8 is approx. 8 Å. In the oxygenation reaction one of the methylene protons will be abstracted in the first and rate-limiting step of the reaction [25,26]; concomitantly, the iron is reduced to the ferrous state [2]. If the methyl group of the substrate is located approx. 6 Å from the iron

atom, like the methyl group of the alcohols (Table III), the methylene protons are relatively close to the iron atom. The observed activity for the polyunsaturated fatty acids with the pentadiene system located at positions *n*-1 to *n*-5 [24] points to a hydrophobic region between a possible methyl binding site and iron. This region may facilitate the binding of the natural *n*-6 substrates. The possibility of a binding of alcohols to the enzyme at a hydrophobic region which serves as substrate binding site has also been suggested by Mitsuda et al. [8]. Fluorescence pertubation studies on lipoxygenase-1 [27] indicate that the active site contains tryptophan residues located in a hydrophobic environment 7 Å from the iron, in general agreement with our distance estimate. Recent NMR studies [18] have shown that the olefinic part of the substrate is bound to the enzyme. Thus, both the terminal alkyl part and the pentadiene system are important for binding. The firm binding of the natural substrate is also reflected by the much lower K_m value (3.3 μM for linoleic acid [28]) compared to the binding constants of the alcohols, which are all in the mM range (Table II).

Acknowledgements

This work was supported by funds from the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO) and the Swedish Natural Science Research Council.

References

1 Tappel, A.L. (1963) in The Enzymes, 2nd Edn. (Boyer, P.D., Lardy, H. and Myrbäck, K., eds.), pp. 275–283, Academic Press, New York
2 De Groot, J.J.M.C., Veldink, G.A., Vliegenthart, J.F.G., Boldingh, J., Wever, R. and Van Gelder, B.F. (1975) Biochim. Biophys. Acta 377, 71–79
3 De Groot, J.J.M.C., Garssen, G.J., Veldink, G.A., Vliegenthart, J.F.G., Boldingh, J. and Egmond, M.R. (1975) FEBS Lett. 56, 50–54
4 Pistorius, E.K., Axelrod, B. and Palmer, G. (1976) J. Biol. Chem. 251, 7144–7148
5 Slappendel, S., Veldink, G.A., Vliegenthart, J.F.G., Aasa, R. and Malmström, B.G. (1981) Biochim. Biophys. Acta 667, 77–86
6 Slappendel, S., Aasa, R., Malmström, B.G., Verhagen, J., Veldink, G.A. and Vliegenthart, J.F.G. (1982) Biochim. Biophys. Acta 708, 259–265
7 Ross, R.T. (1965) J. Chem. Phys. 42, 3919–3922
8 Mitsuda, H., Yasumoto, K. and Yamamoto, A. (1967) Arch. Biochem. Biophys. 118, 661–669
9 Finazzi-Agrò, A., Avigliano, L., Veldink, G.A., Vliegenthart, J.F.G. and Boldingh, J. (1973) Biochim. Biophys. Acta 326, 462–470
10 Galpin, J.R., Tielens, L.G.M., Veldink, G.A., Vliegenthart, J.F.G. and Boldingh, J. (1976) FEBS Lett. 69, 179–182
11 Garssen, G.J., Vliegenthart, J.F.G. and Boldingh, J. (1971) Biochem. J. 122, 327–332
12 Verhagen, J., Veldink, G.A., Egmond, M.R., Vliegenthart, J.F.G., Boldingh, J. and Van der Star, J. (1978) Biochim. Biophys. Acta 529, 369–379
13 Dwek, R.A. (1973) Nuclear Magnetic Resonance in Biochemistry, pp. 174–246, Clarendon Press, Oxford
14 Dahlquist, F.W. and Raftery, M.A. (1968) Biochemistry 7, 3269–3277
15 Raftery, M.A., Dahlquist, F.W., Chan, S.I. and Parsons, S.M. (1968) J. Biol. Chem. 243, 4175–4180
16 Jones, R., Dwek, R.A. and Forsén, S. (1974) Eur. J. Biochem. 47, 271–283
17 Burton, D.R., Forsén, S., Karlström, G., Dwek, R.A., McLaughlin, A.C. and Wain-Hobson, S. (1976) Eur. J. Biochem. 71, 519–528
18 Viswanathan, T.S. and Cushley, R.J. (1981) J. Biol. Chem. 256, 7155–7160
19 Harris, D.C., Gray, G.A. and Aisen, P. (1974) J. Biol. Chem. 249, 5261–5264
20 Taylor, P.W., Feeney, J. and Burgen, A.S.V. (1971) Biochemistry 10, 3866–3875
21 Lanir, A. and Navon, G. (1972) Biochemistry 11, 3536–3544
22 Koenig, S.H. and Schillinger, W.E. (1969) J. Biol. Chem. 244, 6520–6526
23 Egmond, M.R. and Williams, R.J.P. (1978) Biochim. Biophys. Acta 535, 418–422
24 Holman, R.T., Egwim, P.O. and Christie, W.W. (1969) J. Biol. Chem. 244, 1149–1151
25 Hamberg, M. and Samuelsson, B. (1967) J. Biol. Chem. 242, 5329–5335
26 Egmond, M.R., Vliegenthart, J.F.G. and Boldingh, J. (1973) Biochem. Biophys. Res. Commun. 48, 1055–1060
27 Finazzi-Agrò, A., Avigliano, L., Egmond, M.R., Veldink, G.A. and Vliegenthart, J.F.G. (1975) FEBS Lett. 52, 73–76
28 Spaapen, L.J.M., Verhagen, J., Veldink, G.A. and Vliegenthart, J.F.G. (1980) Biochim. Biophys. Acta 618, 153–162