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Comparative Study of Dynamic Structure of Pig and Chicken Aspartate Aminotransferases by Measuring the Rotational Correlation Time

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Abstract. The rotational correlation time of two homologous cytoplasmic aspartate aminotransferase molecules isolated from pig and chicken hearts was obtained by spin-labeling technique. The maleimide and iodoacetamide spin-labels modyfying external SH-groups of a protein were used. In the interpretation of ESR spectra a rotational motion of nitroxide group relative to the protein molecule was taken into account. To determine the macromolecule rotational correlation time two methods of the immobilization of a protein molecule were used: 1) by means of increasing protein solution viscosity and 2) by fixation of the protein molecule on adsorbent. From comparison of experimental and theoretical values of rotational correlation time it was conclude that the both enzymes exhibits an intramolecular flexibility.

Key words: Aspartate aminotransferase – Spin-labels – Correlation time – Intramolecular flexibility

1. Introduction

The aspartate aminotransferase (L-aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1; AAT) is in a focus of intensive studies at a moment. A primery sequence of the pig heart AAT (Ovchinnikov et al. 1973) and a three-dimensional protein structure with a 5 Å resolution in the crystalline state for the chicken heart AAT (Borisov et al. 1978) have already been obtained. However, to understand fully how an enzyme works, one should also know a dynamic structure of a protein molecule in solution. The values of the rotational correlation time (τ) of a macromolecule gives information whether its structure is rigid or flexible.

This paper presents the τ values of two homologous pig and chicken heart AAT molecules obtained by spin-label method as proposed earlier (Dudich et al. 1977; Timofeev et al. 1978). It was found that the value of τ for the chicken AAT

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is 1.6 times smaller than of the pig AAT. Based on comparing experimental and theoretical values τ, a conclusion has been drawn that both homologous enzymes have an internal lability. This conclusion is in a reasonable agreement with a small contact area between subunits in the chicken AAT molecule as has been shown by the X-ray study (Borisov et al. 1978).

2. Materials and Methods

2.1 Proteins and Their Spin-Labeling

The cytoplasmic aspartate aminotransferase (EC 2.6.1.1) was isolated from the pig heart and chicken heart as described previously (Polyanovsky and Telegdy 1965; Khochkina et al. 1978). Each protein concentration was 10–15 mg/ml. The external SH-groups of both aminotransferases was alkylated with the spin-label 2.2.6.6-tetramethylpiperidine-N1-oxyl-4-maleimide (*SLI*). The reaction was allowed to proceed at 4° C for 2 h, in 0.1 M acetate buffer, pH 5.5 and with 10^{-5} M EDTA. The spin-label concentration was 10-times that of the protein, and the ethanol concentration was 3%. The unreacted labels were removed by gel filtration through a Sephadex G-25 (media). The amount of the bound spin-labels was determined by double integration of ESR spectra and independently by the titration SH-groups (Polyanovsky et al. 1972).

Besides, the chicken heart AAT was labeled with the 2.2.6.6-tetramethylpiperidine-N1-oxyl-iodacetamide (*SLII*) with one the same conditions as for *SLI*.

The fixation of the spin-labeled protein on aminobenzylcellulose was performed as described previously (Gurvich et al. 1961).

The ESR spectra of the spin-labeled enzyme in solution at a different viscosity and at 20° C or 0° C were monitored on a E-104A "Varian" spectrometer. The viscosity was varied by adding sucrose.

2.2 Determination of Rotational Correlation Time

The determination of the τ and evaluation of relative rotational freedom of spin-labels are based on the following considerations (Dudich et al. 1977; Timofeev et al. 1978).

The distance between outer wide extrema of ESR spectrum of completely immobilized spin-label is equal $2A_{zz}$, where A_{zz} is the maximal principal value of the electron-nuclear hyperfine tensor $\hat{A}$. (The others two principle values of $\hat{A}$ are designated A_{xx} and A_{yy}). In the common case, when spin-label not completely immobilized the distance between outer wide extrema of ESR spectrum is $2A'_{II}$. This value decreases as spin-label motion becomes more rapid. If spin-label is fixed rigidly to a protein molecule, $2A'_{II}$ depends only on the τ of macromolecule (Shimshik and McConnell 1972). But if macromolecule is completely immobilized ($\tau = \infty$), $2A'_{II}$ reflects only the rotation of spin-label with respect of the protein moiety. In this case the distance $2A'_{II}$ will be

designated as $2\bar{A}_{II}$. In general $2A'_{II}$ depends both on the motion of the macromolecule and on the relative motion of spin-label. Hence, the overall shift $2A_{zz} - 2A'_{II}$ can be expressed as:

$$2A_{zz} - 2A'_{II} = (2A_{zz} - 2\bar{A}_{II}) + (2\bar{A}_{II} - 2A'_{II}) , \tag{1}$$

where $(2A_{zz} - 2\bar{A}_{II})$ is a shift due to relative motion of the spin-label and $(2\bar{A}_{II} - 2A'_{II})$ is a shift due to rotation of the macromolecule.

The ESR spectra of nitroxide radical were simulated on a computor assuming slow isotropic Brownian diffusion of the macromolecule (Shimshik and McConnell 1972) and rapid anisotropic motion of spin-label relative to macromolecule (Dudich et al. 1977). To describe the rapid anisotropic motion of spin-label we used the parameter S (Hubbel and McConnel 1971):

$$S = \frac{\bar{A}_{II} - \bar{A}_{\perp}}{A_{zz} - 1/2(A_{xx} + A_{yy})} , \tag{2}$$

where $\bar{A}_{II}$ and $\bar{A}_{\perp}$ are the averaged parameters which arises from rapid anisotropic spin-label motion with respect to protein moiety. Since

$$1/3(A_{zz} + A_{xx} + A_{yy}) = 1/3(\bar{A}_{II} + 2\bar{A}_{\perp}) = a_0 , \tag{3}$$

where a_0-isotropic hyperfine constant nitroxide radical, then equation (2) can be rewritten as:

$$S = \frac{\bar{A}_{II} - a_0}{A_{zz} - a_0} . \tag{4}$$

This expression more useful than (2) for a_0 is easily available from the experiment than $\bar{A}_I$, A_{xx}, and A_{yy}.

The value of $S = 1$ corresponds to an immobile spin-label relative to the protein molecule, while $S = 0$ corresponds to an entirely mobile spin-label.

The variation of $(2\bar{A}_{II} - 2A'_{II})$ with τ for different values of S is shown in Fig. 1A (right scale). The same figure (Fig. 1B, left scale) shows similar plots for the variation of $\Delta H(-1) = H(\infty) - H(\tau)$ (the shift of a high-field extremum in ESR spectra at variation of the τ value from τ to ∞) with τ. Curves at Figs. 1A and B can be approximated as follows:

$$(2\bar{A}_{II} - 2A'_{II}) \propto \tau^{-\beta} \tag{5}$$

and

$$\Delta H(-1) \propto \tau^{-\beta(-1)} , \tag{6}$$

where β and $\beta(-1)$ vary from 0.74 to 0.86 and 0.67 to 0.74, respectively, when the value of S vary from 1 to 0.53.

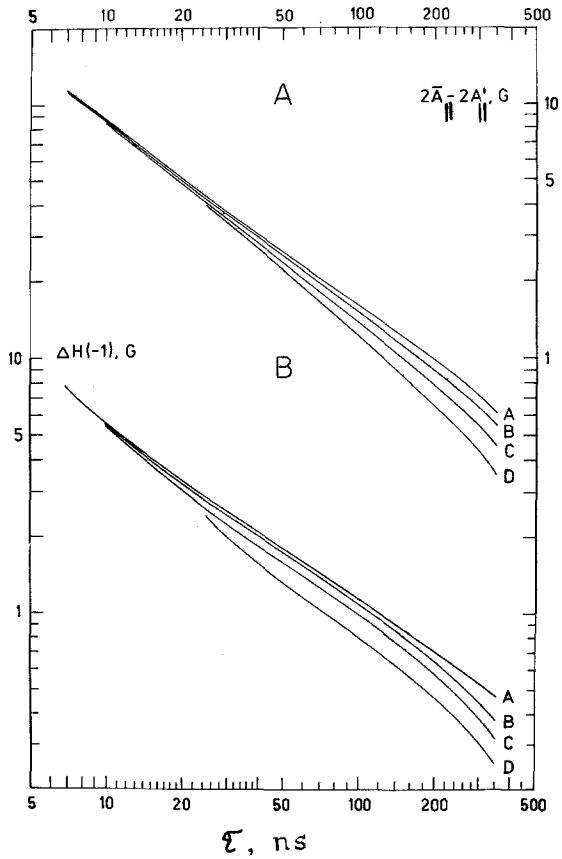

Fig. 1. Nomograms for the determination of τ for the spin-labeled protein molecule at different S on $(2\bar{A}_{II} - 2A'_{II})$, on the right side (**A**), and $\Delta H(-1)$, on the left side (**B**). S equal to: **A** = 1.0; **B** = 0.95; **C** = 0.81, and **D** = 0.53

Value a_0 = 17.1 G for free piperidine nitroxide radical in aqueous solution and value $2A_{zz}$ = 74.5 G for a spin-labeled protein at 77 K were used in this work.

3. Results

3.1 ESR Spectra of Spin-Labeled Aspartate Aminotransferases

Figure 2 shows the ESR spectra of AAT-*SLI* (chicken) and AAT-*SLII* (chicken)[1]. The spectrum of AAT-*SLI* (pig) has a similar line shape as the spectrum AAT-*SLI* (chicken). The AAT-*SLI* spectrum at 20° C and 0% sucrose (see Fig. 2a) has clearly distinguishable outer extrema.

In order to separate effects of the macromolecule tumbling and the relative label motion upon the position of the outer wide peaks, AAT-*SLI* (chicken) was

1 AAT-*SLI* and AAT-*SLII*, aspartate aminotransferases labeled at SH-groups with spin-labels *SLI* and *SLII*, respectively

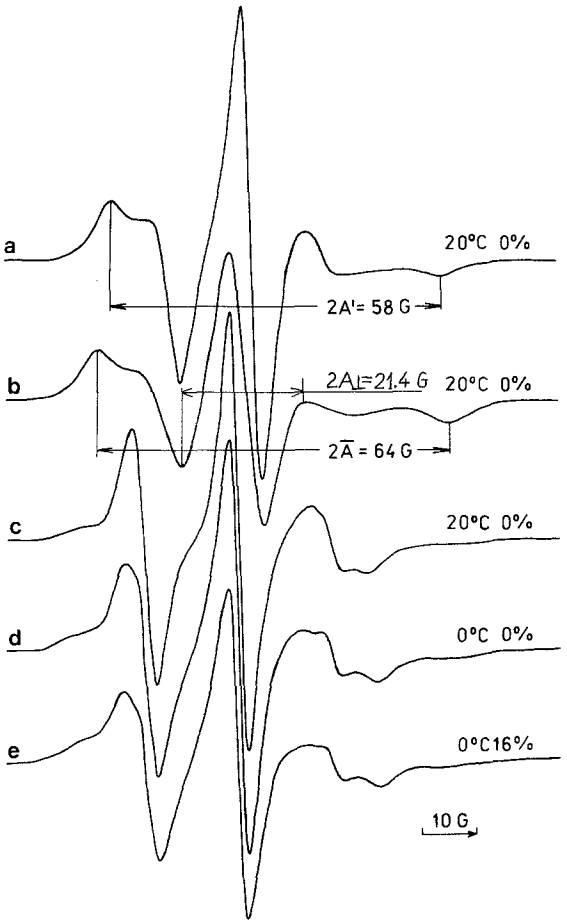

Fig. 2. ESR spectra of chicken AAT-*SLI* and AAT-*SLII*: **a** = AAT-*SLI* at 20° C and 0% sucrose; **b** = AAT-*SLI* coupling to an adsorbent at 20° C and 0% sucrose; **c** = AAT-*SLII* at 20° C and 0% sucrose; **d** = AAT-*SLII* at 0% and 0% sucrose; **e** = AAT-*SLII* at 0° C and 16% sucrose

immobilized by the aminobenzylcellulose adsorbent. With a general spectrum form retaining, one can clearly see (Fig. 2b) a shift of outer peaks centrewise (and inner peaks edgewise) at the spectrum obtained in this way.

Alkylation of a highly reactive external SH-group of the chicken enzyme by *SLII* results in a ESR spectrum shown in Fig. 2c. The spectrum corresponds to a moderate immobilized spin-label since the chemical structure of *SLII* allows for a greater rotational freedom of the nitroxide group than the structure of *SLI*. Decreasing the temperature to 0° C, leads to an additional immobilization of a *SLII* and results only in an appearance of a well resolved high-field outer peak (Fig. 2d). Because of this, determining the value of τ in a case of ATT-*SLII* has been carried out at 0° C.

The number of covalently-bound labels per enzyme monomer proved to be such that *SLI* and *SLII* had to be bound with two external SH-groups of the pig enzyme (Polyanovsky et al. 1972) and one group of the chicken enzyme (Khochkina and Torchinsky 1975).

3.2 *Quantitative Analysis of ESR Spectra of Spin-Labeled ATT in Solution*

Figure 3 shows the $2A'_{II}$ value versus $(T/\eta)^{0.76}$ for AAT-*SLI* (chicken and pig) and the $\Delta H(-1)$ value versus $(T/\eta)^{0.74}$ for AAT-*SLII* (chicken). At 0–40% sucrose experimental relations correspond to theoretical ones [see Eqs. (5) and (6)] and give $2\bar{A}_{II}$ (see Fig. 3A) and $H(\tau = \infty)$ (see Fig. 3B) at $T/\eta = 0$. Parameters needed to compute the value of τ on the basis of graphs in Fig. 3 are presented in Table 1.

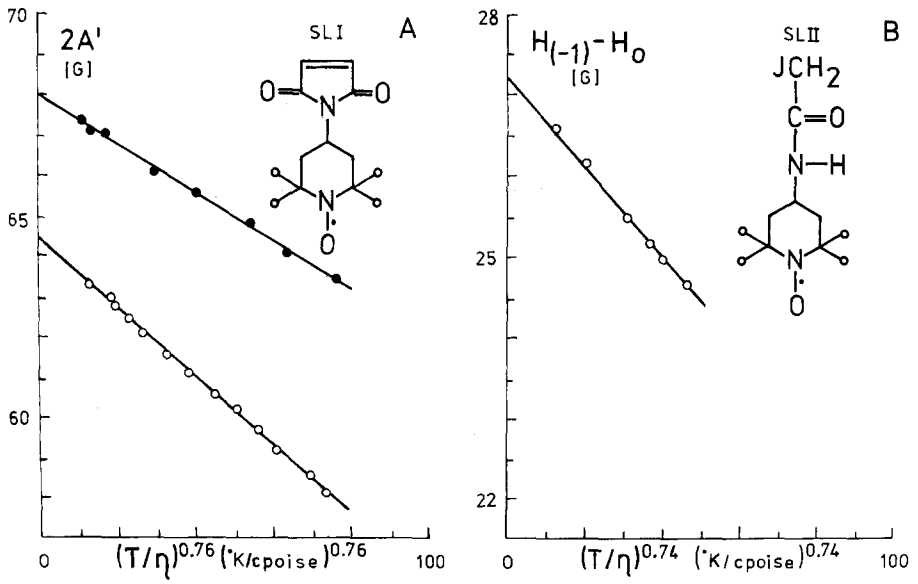

Fig. 3. Experimental verifications of Eqs. (4) and (5); **A** = dependence $2A'_{II}$ on $(T/\eta)^{0.76}$ for AAT-*SLI* (pig) (●) and AAT-*SLI* (chicken) (○); **B** = dependence $\Delta H(-1)$ on $(T/\eta)^{0.74}$ for AAT-*SLII* (chicken)

Table 1. Parameters of ESR spectra and τ values of spin-labeled aminotransferase molecules

Spin-labeled proteins	°C	$2\bar{A}_{II}$ [G]	S	$(2\bar{A}_{II} - 2A'_{II})^a$ [G]	$\Delta H(-1)$ [G]	τ [ns]	$\tau^{20°C b}$ [ns]
AAT-*SLI* (pig) (free in solution)	20	68.0	0.91	4.2	–	23.0	23.0
AAT-*SLI* (chicken) (free in solution)	20	64.5	0.81	6.6	–	14.5	14.5
AAT-*SLI* (chicken) (couping on adsorbent)	20	64.0	0.81	–	–	–	–
AAT-*SLII* (chicken) (free in solution)	0	55.0	0.53	–	2.5	22.5	14.5

[a] $2\bar{A}_{II}$ was measured using the plots in Fig. 3 at $(T/\eta) = 300$ corresponding to 20° C and 0% sucrose

[b] The value of τ reduce to a normal conditions at 20° C and 0% sucrose

The $2\bar{A}_{II}$ value for AAT-*SLI* (pig) equal to 68 *G* is greater than the $2\bar{A}_{II}$ = 64.5 *G* for AAT-*SLI* (chicken) indicating the more restricted label rotational capacity with respect to the protein moiety. To determine the values of τ of AAT-*SLI* (pig), AAT-*SLI* (chicken) and AAT-*SLII* (chicken) the curves A and B in Fig. 1A and D in Fig. 1B were used for $S = 0.91$, $S = 0.81$ and $S < 0.53$, respectively.

Different inclinations of stright lines in Fig. 3A yields two values of τ equal to 23 ns for AAT-*SLI* (pig) and 14.5 ns for AAT-*SLI* (chicken). The same time 14.5 ns has been obtained for AAT-*SLI*.

As it is seen from Fig. 2a and b, both inner and outer peaks of AAT-*SLI* ESR spectra were resolved. We measured separation of the inner hyperfine extrema ($2A'_I$) versus η. As a result, we obtained $2\bar{A}_{\perp} = 21.1$ G which along with $2\bar{A}_{II}$ = 64.5 G gave $a_0 = 17.8$ G.

3.3 ESR Spectrum of Chicken AAT-SLI Fixed on Adsorbent

Figure 2b shows the ESR spectrum of AAT-*SLI* (chicken) immobilized on aminobenzylcellulose. The separation between outer peaks is equal to 64 G. This value is close to the value of $2\bar{A}_{II} = 64.5$ G, which obtained by extrapolated procedure (see Fig. 3A and Table 1). Thus, we can conclude the rotation of the AAT-*SLI* molecule is completely immobilized ($\tau > 10^{-6}$ s) and this in turn provides a good way to measure a_0, by measuring $2\bar{A}_{II}$ and $2\bar{A}_I$ directly from the ESR spectrum in Fig. 2b. The obtained in such a way the value a_0 was equal to 17.8 G.

4. Discussion

The results obtained in our work are a consequence of a new theoretical and experimental procedure of estimating τ for the spin-labeled protein molecule with account for the relative nitroxide mobility (Dudich et al. 1977). That approach have been already tested on a number of different molecular weight globular proteins with various spin-labels (Dudich et al. 1977; Timofeev et al. 1978; Sykulev et al. 1979; Timofeev et al. 1979; Dudich et al. 1979). The basic requirement to application of this method is as follows: anisotropic relative-to-protein nitroxide motion must be rapid enough ($\tau_{\text{label}} \leqslant 10^{-9}$ s). This requirement is valid, as is seen from the linearity of graphs in Fig. 3. First, the τ of a macromolecule must increase linearily with incrasing of the solution viscosity following the Einstein-Stokes law. Second, the diagonal elements partial averaging of hyperfine tenzor is constant up to about 40% sucrose.

It should be note, the analogy between a spin-label method and a fluorescence depolarization method is complete. The dye rotates rapid relative to the macromolecule while the overall motion is that of the macromolecule to which it is attached is slow (Wahl and Weber 1967).

As noted above the coupling of spin-labeled protein to an adsorbent is a method which manifests only relative mobility of the label and provide evidence

that there is fast motion of the spin-label of about the protein moiety. As a result of this, $2\bar{A}_{II} = 64$ G (as seen from Fig. 2b) has actually proved to be near equal to the extrapolated value of $2\bar{A}_{II} = 64.5$ G (see Table 1). The small difference in the $2\bar{A}_{II}$ values is due to the chemical modification by attaching covalently the enzyme to an insoluble support, which induces some local conformational change of an immediate environment of the spin-label.

The a_0 value of bounded *SLI* equal to 17.8 G which we have determined by two methods (see Sect. 3.2 and 3.3) close to $a_0 = 17.1$ G as for the nitroxide radical in water and indicate that the nitroxide is exposed in the solution.

As seen from Table 1, the value of τ for the chicken-AAT differs from that for the pig-AAT, but, in turn, both correlation times are also differ from $\tau = 45$ ns, as predicted for a rigid sphere of the mol.wt. 95,000 (Polyanovsky et al. 1970). It follows that the flexibility of the AAT molecule seems to exist. The sharp difference of above times for the two homologous enzymes can be easily understood. Without chicken heart AAT primery sequence data, a homology may be suggested between the external fast reacting chicken enzyme SH-group and external pig enzyme SH-group (Cys-45 or Cys-82). However, this would mean a drastical difference in a three dimensional structure of these two proteins. But there is a contrary opinion that the quarternary and tertiary of both enzymes are identical (Capasso et al. 1979), and two pig enzyme external SH-group belong to a bigger relaxating part of the monomer, and one chicken enzyme external SH-group is in the smaler part of it.

By the spin-label method we have estimated the rotational relaxation of monomer AAT parts structurally organized in a dimer. Thus, we have come across the evidence of the intermolecule flexibility both for the enzyme dimer, and enzyme monomer of the two homologous aspartate aminitransferases.

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References

Berliner LJ (1974) Application of spin labeling to structure-conformation studies of enzymes. Prog Bioorg Chem 3: 1–80

Borisov VV, Borisova SN, Kachalova GS, Sosfenov NI, Vainstein BK, Torchinsky YuM, Braunstein AE (1978) Three dimensional structure at 5 Å resolution of cytosolic aspartate transaminase from chicken heart. J Mol Biol 125: 275–292

Capasso S, Garzillo AM, Marino G, Mazzarella L, Pucci P, Sannia G (1979) Mitochondrial bovine aspartate aminotransferase. Prelimenary sequence and crystallographic data. FEBS Lett 100: 351–354

Dudich IV, Timofeev VP, Volkenstein MV, Misharin AYu (1977) Macromolecule rotational correlation time measurement by ESR method for covalently bound spin-label. Mol Biol (USSR) 9: 531–538

Dudich IV, Timofeev VP, Volkenstein MV (1979) Simple analysis of ESR spectra of spin-labeled macromolecules with account of fast anisotropic rotation of a spin-label and slow diffusion rotation of a macromolecule. In: Kundla E, Lippmaa E, Saluvere T (eds) Magnetic resonance and related phenomena. Proceedings of the XXthe Congress AMPERE, Tallin, USSR. Springer, Berlin Heidelberg New York

Gurchiv AE, Kuzovleva OB, Tumanova AE (1961) Production of protein-cellulose complexes (immunosorbents) in the form of suspensions able to bind great amounts of antibodies. Biokhimia USSR 26: 934–942

Hubbel WL, McConnell HM (1971) Molecular motions in spin-labeled phospholipids and membranes. J Am Chem Soc 93: 314–326

Khochkina VM, Torchinsky YuM (1975) Aspartate aminotransferase from chicken heart cytosol. Characterization of SH-groups. Biokhimia USSR 40: 812–818

Khochkina VM, Azaryan NV, Mechanic ML, Zapomerdina LN, Sinitsina HI, Egorov CA, Torchinsky YuM (1978) Improved procedure for the purification of aspartate transaminase from chicken heart cytosol. Characterization of the enzyme. Biokhimia USSR 40: 1478–1484

Ovchinnikov YuA, Egorov CA, Aldanova NA, Feigina MYu, Lipkin VM, Abdulaev NG, Grishin EV, Kiselev AP, Modyanov NN, Braunstein AE, Polyanovsky OL, Nosikov VV (1973) The complete amini acid sequence of cytoplasmic aspartate aminotransferase from pig heart. FEBS Lett 29: 31–34

Polyanovsky OL, Telegdy M (1965) On the functions of thiole groups of aspartate-glutamate transaminase. Biokhimia USSR 30: 174–182

Polyanovsky OL, Zagyansky YuA, Tumerman LA (1970) Molecular parameters and the structure of aspartate-transaminase and its subunits. Mol Biol USSR 5: 458–470

Polyanovsky OL, Timofeev VP, Shaduri MI, Misharin AYu, Volkenstein MV (1972) Conformational change of aspartate aminotransferases in the region of Cys-45 residue observed by means of spin-label. Biochim Biophys Acta 327: 57–65

Shimshik EJ, McConnell HM: Rotational correlation time of spin-labeled α-chemotrypsin. Biochem Biophys Res Commun 46: 321–326

Sykulev YuK, Timofeev VP, Nezlin RS, Franêk F (1979) Spin-label study of segmental flexibility of anti-hapten antibodies. Precipitating pig anti-Dnp antibody is more flexible than non-precipitating. FEBS Lett 101: 27–30

Timofeev VP, Dudich IV, Sykulev YuK, Nezlin RS (1978) Rotational correlation times of IgG and its fragments spin-labeled at carbohydrate of protein moieties. Spatially fixed position of the F_c carbohydrate. FEBS Lett 89: 191–195

Timofeev VP, Dudich IV, Sykulev YuK, Nezlin RS, Franêk F (1979) Slow conformational change in anti-dansyl antibody as a consequence of hapten binding. Demonstration by ESR spectra. FEBS Lett 102: 103–106

Wahl P, Weber G (1967) Fluorescence depolarization of rabbit gamma globulin Conjungates. J Mol Biol 30: 371–382

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