Chapter 13

Quantitative Determination of Thiol Status of Proteins and Cells by Nitroxyl Biradical ·RS-SR·

Lev Weiner

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/45620

Dedicated to the memory of Leonid Volodarsky

1. Introduction

Sulfhydryl (thiol, SH) groups of proteins and of low-molecular weight compounds, such as glutathione (GSH) and cysteine (Cys) play important roles in numerous biological processes. In the last decades, interest in the redox state of SH groups in proteins has grown because thiol-disulfide exchange has been found to play an important role in protein folding and to influence protein stability (1-3).

In cells, the ratio/equilibrium between oxidized and reduced forms of glutathione and between cysteine and cystine (main cells antioxidants) affect thiol balance and redox status of cells and proteins (4). In general, spectroscopic and chromatographic methods are used for quantitative determination of low-molecular thiols and sulfhydryl groups in proteins. Optical methods are employed to detect absorption or fluorescence, which appears on results of interaction between reagents and free SH groups. However, samples must be optically transparent, so preliminary homogenation and centrifugation of biological samples and other procedures are necessary (4). Chromatographic methods, especially HPLC (4), cannot be used for express analysis of thiol status of biological samples.

Among the optical methods for determining the free thiol groups the method proposed by Ellman (5) is definitely in the first place. This approach is based on the thiol-disulfide exchange reaction between the disulfide containing reagent (5,5'-dithiobis-(2-nitrobenzoic acid, DTNB), **ES-SE**, and the free thiol, **SH-T**:

$$\text{ES-SE} + \text{SH-T} \rightarrow \text{ES-ST} + \text{ES}^- \quad (1)$$

The resulting product, mono-thiol, $\mathbf{ES}^-$, has a characteristic optical spectrum (λ_{max}= 412 nm) with a known extinction coefficient, ε=14 150 $M^{-1}cm^{-1}$. It is the simplicity of this method that has determined its widespread use (more than 13,500 citations for 50 years!). However, this

method suffers from all the drawbacks typical for other optical methods: the impossibility of measuring in a colored, scattering, and turbid media, i.e. in real biological systems. In addition, the sensitivity of this method is often insufficient.

In 1987 we had a project including reversible modification of SH-group in NADPH-cytochrome P-450 reductase. We decided to get a paramagnetic analogue of the Ellman reagent, stable nitroxyl biradical, containing disulfide bond (6). We hoped that, if successful, the biradical would enter the free thiol/biradical thiol-disulfide exchange reaction, which could be followed by ESR. Our colleagues, Vladimir Martin and Tatyana Berezina from the team of Prof. Leonid Volodarsky (Institute of Organic Chemistry, Novosibirsk), synthesized biradical for the task (6,7)

In contrast to the known at that time disulfide containing spin label, [(1-Oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate],MTSSL, (8), our biradical allowed us to kill two birds with one stone: (a) to measure the kinetics of chemical modification of available SH groups in the protein (by appearance of free radical in solutions) and (b) using a traditional technique, i.e. gel- filtration or dialysis, to get the spin-labeled protein after incubation with our probe.(See eq.2 and Fig. 1))

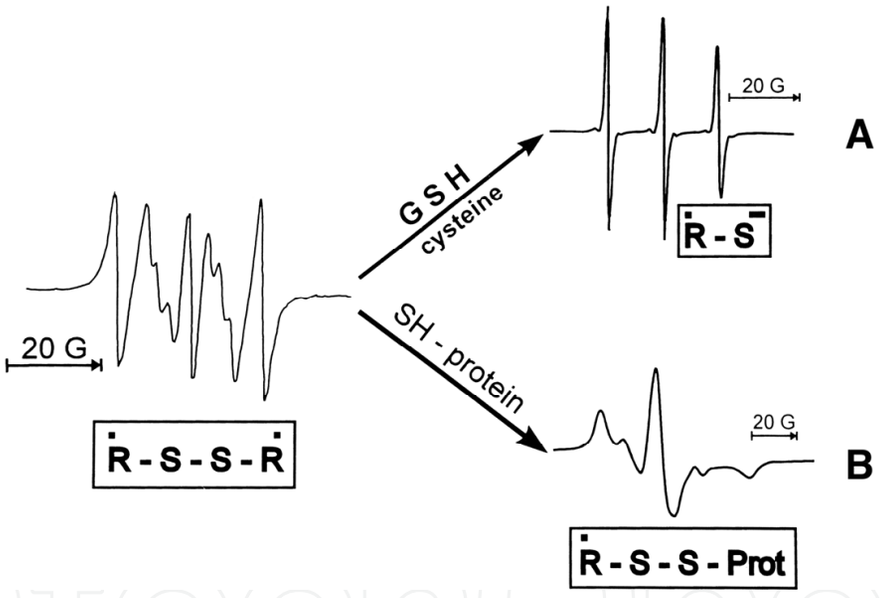
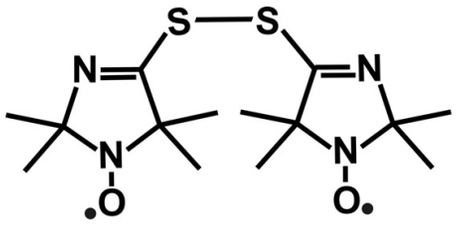


Figure 1. ESR spectra of biradical and its reaction products. (**A) –** the monoradical formed on a reaction with low-molecular weight thiol or free SH group in the protein and (**B) -** the immobilized radical formed on a reaction with protein-linked SH group.

This approach combines advantages of the methodology developed by Ellman (5) that makes use of thiol – disulfide exchange reaction (see eq. 1) and of ESR, that provides high sensitivity and possibility of carrying out work in colored and/or turbid and scattering media, such as cells, tissue culture, blood, etc.

2. Use of SNRs for determination of Thiol status in cells

For this purpose the symmetrical biradical containing disulfide bond, bis(2,2,5,5-tetramethyl-3-imidazoline-1-oxyl-4-il)-disulfide, ·**RS-SR**· was synthesized:



The observed ESR spectrum of ·**RS-SR**· is typical for symmetrical biradical with intermediate character of exchange between two unpaired electrons (9) (see fig.2A).

In the presence of a free thiol group the reaction of thiol-disulfide exchange takes place:

$$\bullet\mathbf{RS-SR}\bullet + \mathbf{HS-A} \rightleftarrows \bullet\mathbf{RS-SA} + \bullet\mathbf{R-SH} \quad (2)$$

$$\bullet\mathbf{RS-SA} + \mathbf{HS-A} \rightleftarrows \mathbf{AS-SA} + \bullet\mathbf{R-SH} \quad (3)$$

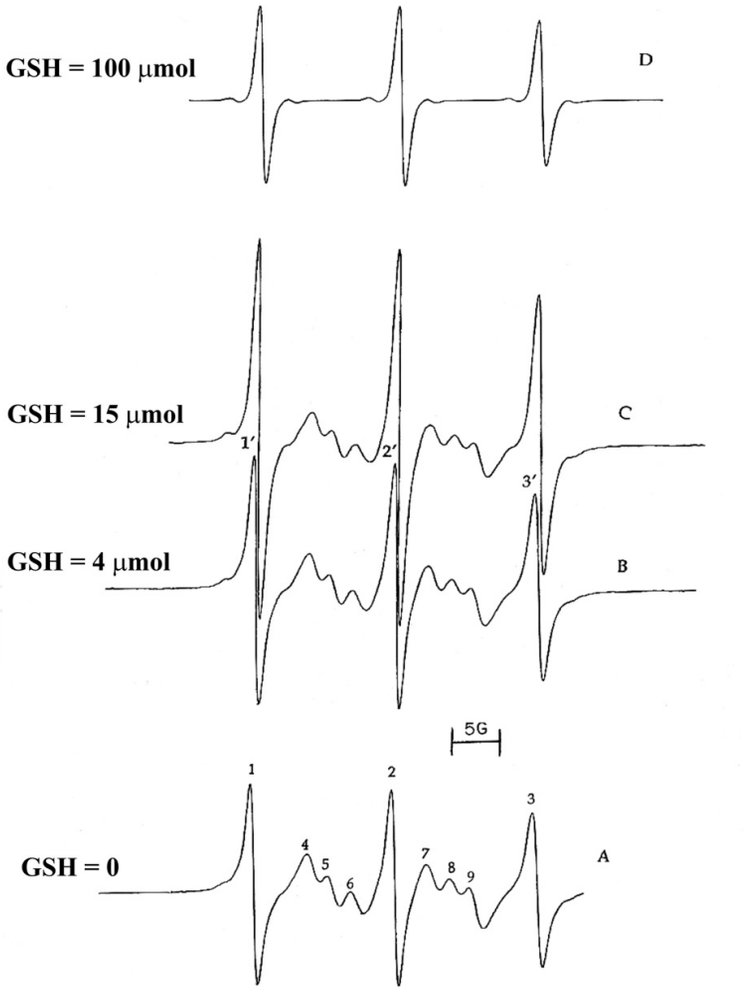


Figure 2. The effect of GSH on ESR spectra of ·**RS-SR**· (100 μM) in PBS, pH=7.5. Spectra **A, B, C** were carried out at: *gain* 5^x10^4, modulation amplitude 1 G, microwave power 10 mW; Spectrum **D**- *gain* 3.2^x10^3, modulation amplitude 1 G, microwave power 10 mW.

The exchange integral, J, was estimated: J = 3.6 a_N (9,10). The absence of any change in the ESR spectrum up to 80°C can be interpreted in terms of existence of a single average conformation of ·**RS-SR**· in solution.

Figure 2 shows the effect of reduced glutathione, GSH, on the ESR spectrum of ·**RS-SR**· : with increasing GSH concentration, the biradical spectral components (1,2,3,4,5,6,7,8,9) decrease with simultaneous increase of monoradical components (1',2'3'). Thus nine broadened components of biradical decrease with concomitant appearance of three narrow components of two monoradicals as a result of the sequential reactions:

Note that the integral intensity of the ESR spectrum of ·**RS-SR**· remains unchanged. The peak intensities of the monoradical components **(·R-SH)** resulting from reactions (2,3) are about 17-fold higher than those of the corresponding biradical components (whose position in the field coincides with that of monoradicals). These phenomena provide the physical basis for the proposed method.

Four reviews give a detailed description of the physical and chemical background for the practical use of the biradical ·**RS-SR**· for thiols meaurements (10-13).

The proposed methodology allowed quantitative assessment of glutathione concentrations in mouse erythrocytes (7), in hamster ovary cells (10,14) and various types of malignant cells (15,16). ·**RS-SR**· is a hydrophobic molecule and , therefore, can easily cross biological membranes and penetrate into cells.

In contrast to conventional methods, our approach is non-invasive and suitable for work with *intact* cells and tissues. It is also extremely sensitive, permitting determination of GSH concentrations in as few as 100 cells (11,14). The method was also used successfully to measure GSH in an isolated reperfused heart (11). Using the biradical ·**RS-SR**· in conjunction with the spin trap DMPO, we were able to demonstrate that the efficacy of oxygen radical generation, stimulated by redox active quinones, correlated with GSH levels and the induction of expression of GSH transferase in cancer cells (15,16). The biradical method was also successfully applied to the monitoring of GSH levels in cancer cells treated with allicine, an active component of garlic, which can arrest the proliferation of cancer cells (17).

The biradical method was also used for direct determination of the catalytic activity of acetylcholinesterase in homogenates of the heads of individual larvae of the bollworm *Heliothis armigera,* following the rate of hydrolysis of acetylthiocholine by monitoring reduction of biradical by the thiocholine produced , according to eq.2(10,18).

Note that synthesis of new disulfide containing SNR is still in progress (19,20). Using the new disulfide containing biradical, the glutathione level (by L-band ESR spectrometer) in tumors in nude mice was measured. This "improved" biradical contains N-15 where deuterium substitutes for hydrogen atoms. This approach enhances the method sensitivity (20).

3. Determination of availability of Thiol groups in proteins

Traditionally, both alkylation and acylation spin labels have been used for chemical modification of proteins using SNRs. After incubation of nitroxyl radical with protein, the

modified protein is separated from the free SNR by gel filtration, dialysis or precipitation. The use of biradical ·**RS-SR**· permits direct measurement of the rate of protein modification by the monitoring the appearance of the free monoradical, ·**R-SH,** in solution, (Fig. 1,eq. 2) thereby providing "visible" information about the rate of thiol-disulfide reaction (eq. 2) and, consequently, about the availability of the thiol group in the protein.

3.1. ESR study of the alcohol dehydrogenase free SH groups

Figure 3 illustrates the kinetics of chemical modification of the thermophilic alcohol dehydrogenase *from Thermoanaerobacter brockii* (TBADH) by biradical ·**RS-SR**· (10, 21). The high reaction rate suggests that when modified, the free thiol group is highly accessible. Modification of TBAD by [2-^{14}C] iodoacetic acid and identification of the labeled peptide indicated that the thiol group labeled was that of Cys 203. In the presence of coenzyme, $NADP^+$, the rate of modification falls (Fig.3, line C) providing evidence that $NADP^+$ interacts with Cys 203. The kinetics of chemical modification of TBADH after removal of Zn^{2+} from its active site by treatment with phenanthroline were almost two-fold higher than for the native enzyme (Fig.3, curve B). As follows from HPLC analysis of the radio-labeled peptide in apo-TBADH, Cys 37, which serves as a ligand for Zn^{2+} in the active site, is available for modification. Using apo-TBADH double-labeled at both Cys 203 and Cys 37 with biradical,

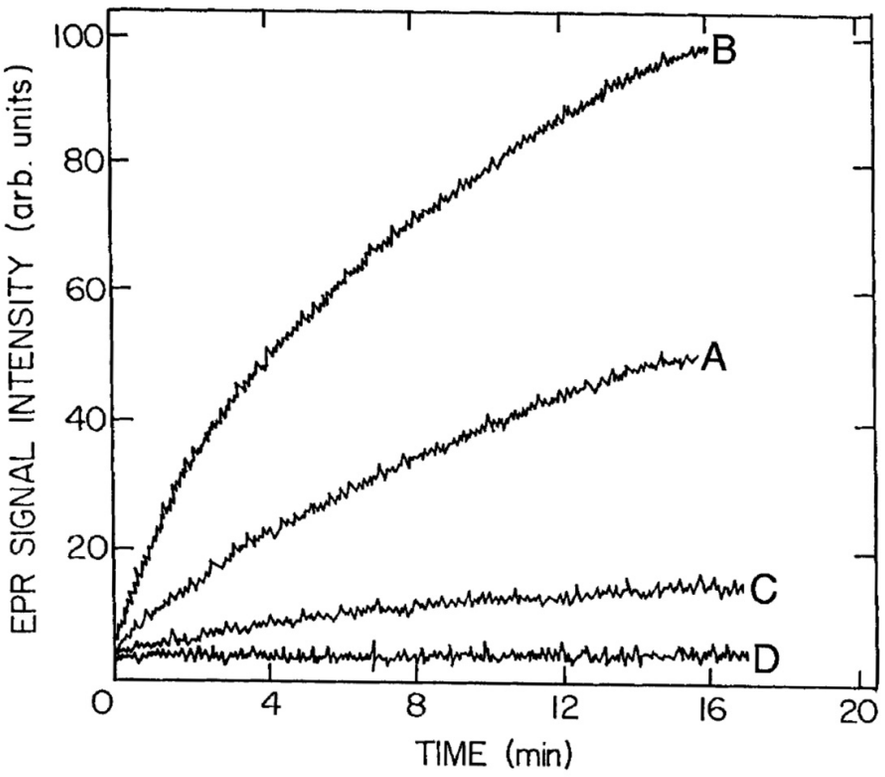


Figure 3. Kinetics of modification of TBADH by biradical ·**RS-SR**·. The peak intensity of the monoradical component, ·**R-SH** (released to solution as a result of reaction between biradical and TBADH (see eq.2)) was monitored. **A**- native TBAD; **B**-apo-TBAD; **C**-TBADH in the presence of $NADP^+$; **D**-TBADH pretreated with *p*-chloromercury benzoate. (from ref. 21)

we were able to estimate the distance between radicals covalently bound at these two cysteines (r~ 10 Å). Later, our colleagues in Weizmann Institute obtained an X-ray structure of TBADH at 2.5 Å resolution (22). The 3D structure has revealed that Cys 203 is indeed a surface residue which is occluded by the coenzyme $NADP^+$ (Fig.4)

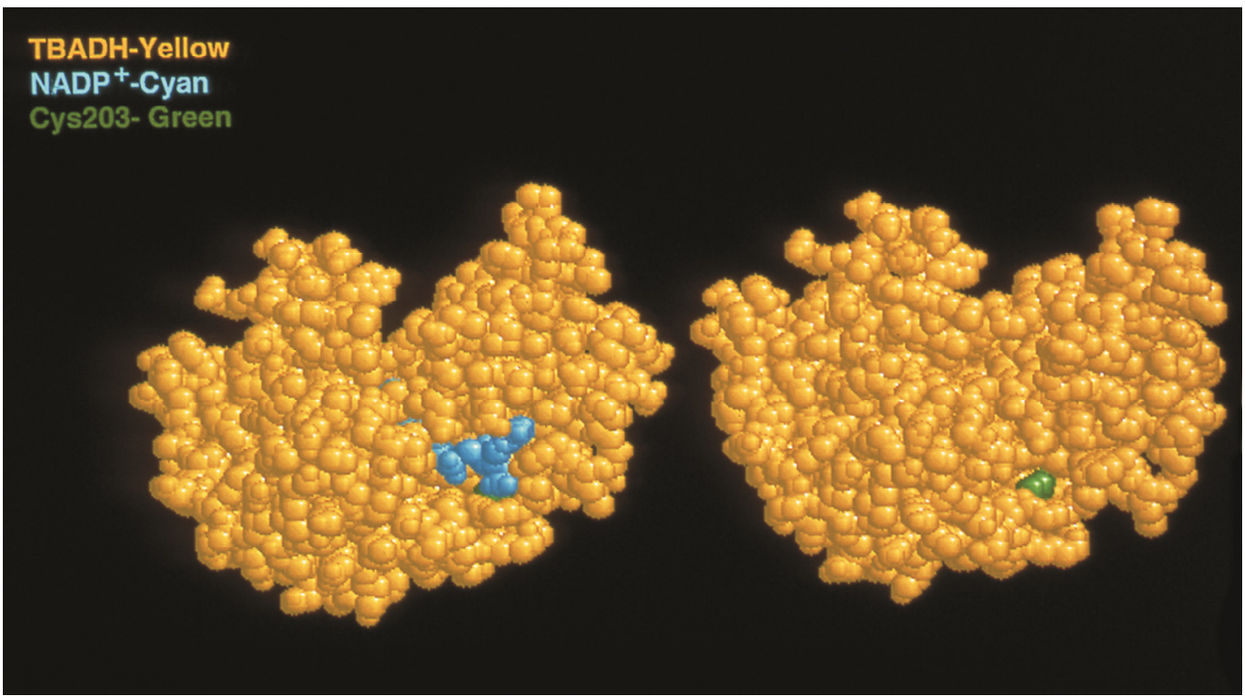
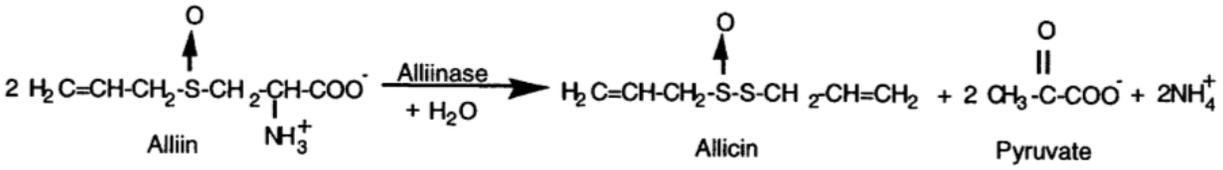


Figure 4. Representation of the monomer of TBADH (PDB entry 1ykf). Individual residues are represented as spheres colored in yellow, the $NADP^+$ cofactor and Cys 203 are colored in cyan (left) and green (right), respectively.

3.2. ESR study of the alliinase's SH groups

Alliinase (Cys sulfoxide lyase, alliin lyase, C-S lyase; EC 4.4.1.4) from garlic (Allium sativum) is an enzyme that uses pyridoxal-5'-phosphate (PLP) as a cofactor to catalyze the conversion of a nonprotein amino acid alliin , Sally cysteine Sulfoxide, to allicin (diallyl thiosulfinate), pyruvate, and ammonia, as shown in the following scheme:



Scheme 1.

Allicin, a product of the enzymatic reaction of alliinase with alliin, is a well-characterized, biologically active compound of garlic. It is responsible for the pungent odor and for a variety of biological effects attributed to garlic preparations, including antimicrobial, anticancer, antiatherogenic, and other activities (23, 24)

Incubation of native alliinase either with 4,4′-dithiodipyridine (DTP) or with 5,5′-dithio-bis-(2-nitrobenzoic acid) (Ellman reagent) in the presence of 6M guanidine- HCl provided evidence for the existence of two free cysteine residues in the alliinase molecule.

To identify the free cysteine residues, alliinase was modified by treatment with N-(4-dimethylamino-3, 5-dinitrophenyl) maleimide (DDPM) and digested with trypsin, chymotrypsin or pepsin (27). Peptides in digests containing the nitrophenyl chromophore were separated and detected on a 360- nm absorbance profile using reversed-phase HPLC. By analyzing the trypsin and chymotrypsin digests, we were able to identify a single (but different) Cys-containing peptide in each case. In the case of trypsin, it was a peptide containing a sequence with Cys220, and in the case of chymotrypsin the peptide contained the sequence with Cys350 (27). Treatment with pepsin made it possible to identify both of these free cysteine residues simultaneously in one digest. These experimental findings (predating the alliinase structure determination) provided direct confirmation that the two free thiols in the alliinase molecule (27).

Using ESR spectroscopy, we examined the availability of the free —SH groups of alliinase for chemical modification with the disulfide containing biradical ·**RS-SR**· (27). The rate of the thiol–disulfide exchange reaction was monitored by ESR assay of the monoradical **·R—SH** released in this reaction, according to Eq. 2.

Figure 5 shows the increase in peak intensity of the ESR signal for the reaction between the biradical and the native alliinase. These data demonstrate that the kinetics of modification occur at two different rates. Pretreatment of alliinase with *p*-chloro mercury benzoate dramatically inhibited the modification kinetics (data not shown). Figure 6 shows the ESR spectrum of alliinase modified by the biradical (after 5 h of incubation followed by removal of the excess reagent by gel filtration) at 120 K. This spectrum is typical of a nitroxyl stable radical in a frozen solution. The degree of modification obtained by double integration of the ESR spectrum was 1.61 ± 0.15 per subunit of alliinase. To estimate the distance between two labeled cysteine residues, we used the empirical parameter d_1/d (see Fig.6), which characterizes the dipole–dipole interaction between unpaired electrons of two nitroxyl groups, as proposed by Kokorin *et al* (28) and commonly used to estimate the distance between two radicals covalently bound to proteins (29, 30). In the absence of dipole–dipole interactions between the radicals, a value $d_1/d < 0.4$ is expected. The value obtained for d_1/d obtained in our experiment was 0.38, and the distance between the labeled cysteines Cys 220 and Cys 350 was estimated to be larger than 22 Å.

Alliinase has been crystallized and its three-dimensional structure solved (25-27). The enzyme is a homodimeric glycoprotein belonging to the fold-type I family of PLP-dependent enzymes.

As shown earlier with biochemical methods, the enzyme subunit contains two free thiols: Cys220 in the PLP-binding domain 2 and Cys350 in the C-terminal part of domain 1 (C7 and

C8, respectively) (Figs7 and 8) located relatively far from the active site and from the substrate-binding area. As shown in Figure 8(A), Cys220 is located on the surface of the alliinase molecule, while Cys350 is in a more buried location but is still water-accessible. The free thiol groups of Cys220 and Cys350 have different relative orientations with respect to each other (Fig. 8B), which might affect their chemical modification rates by ·**RS-SR**· (see Fig. 5). Distances between all the cysteines involved in disulfide bonds and the free thiols in the alliinase dimer range between 15 and 68 Å, and do not allow rearrangement of disulfide bonds in the native state.

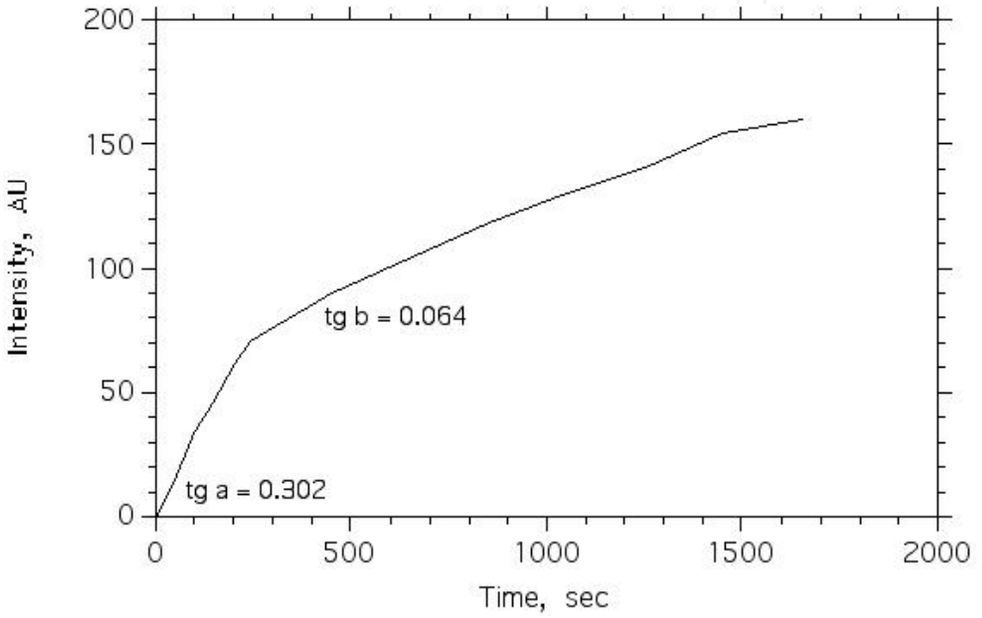


Figure 5. Kinetics of the nitroxyl biradical modification of alliinase. Peak intensity of the ESR spectrum of the monoradical **(·R-SH)** component that was released into solution as a result of the thiol–disulfide exchange between SH groups of alliinase and the biradical ·**RS-SR**· (see eq. 2) (from ref. 27)

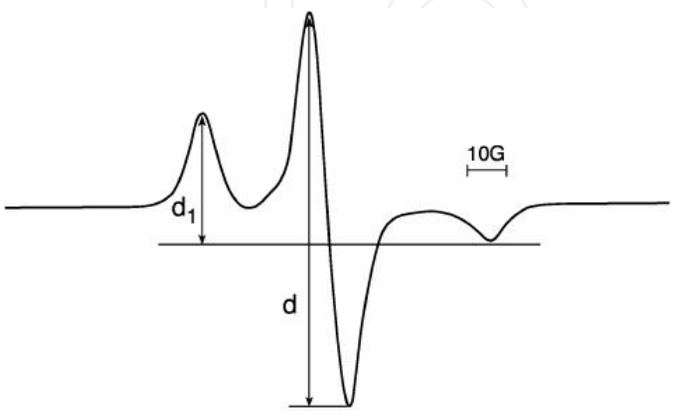
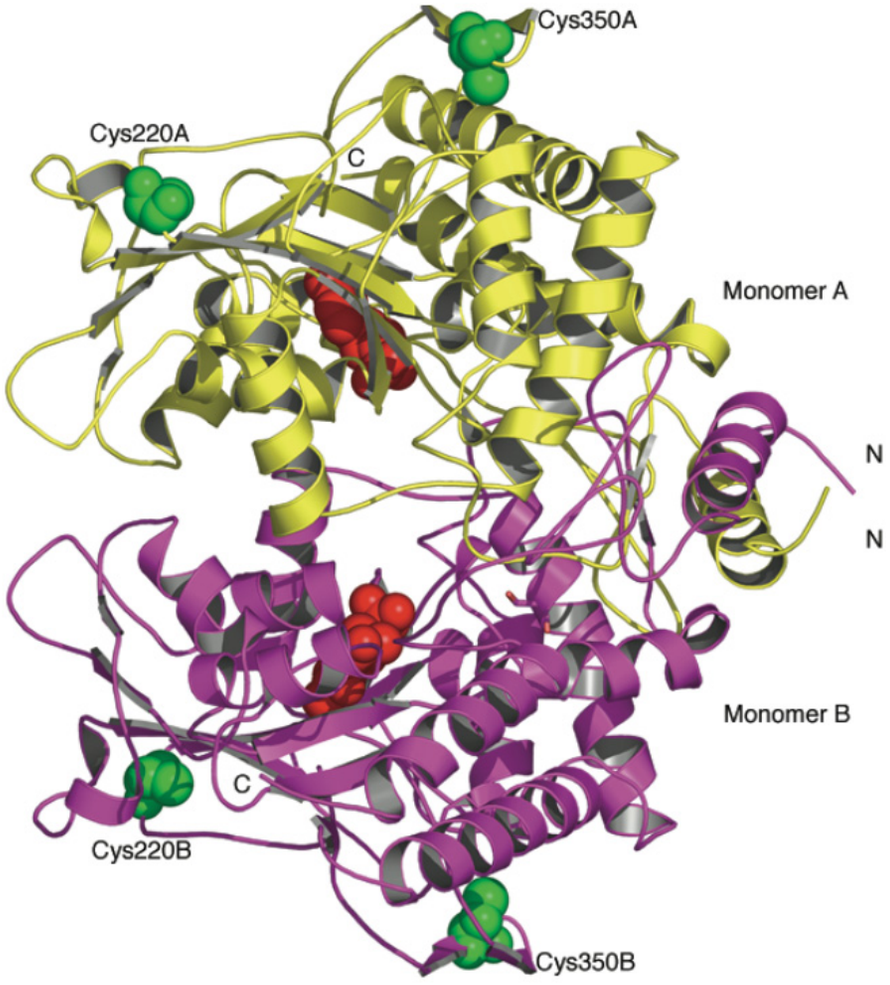


Figure 6. ESR spectrum of the alliinase-biradical conjugate. The conjugate (12 μM) was measured in a PBS/glycerol (70/30) mixture at 120 K. ESR conditions: microwave power, 10 mW; modulation amplitude, 1.25 G;



Dimeric structure of alliinase is depicted in the illustration. Yellow and magenta indicate monomers A and B, respectively. Pyridoxal-5′-phosphate groups are shown as red spheres. The dimer is rotated 180^0 around a vertical axis with respect to A. Free cysteines are shown as green spheres

Figure 7. Distribution of cysteines in a monomer of alliinase from garlic (*Allium sativum*). (from ref. 27)

4. Reversible modification of Thiol groups in proteins

Free thiol groups, whether intrinsic or introduced by site-directed mutagenesis are convenient targets for introduction of stable nitroxyl radicals, SNRs, into proteins. Now, this approach, named site directed spin labeling is very popular, because it can give information about structure (mobility) of different parts of the protein globule (31-33). "Classical" SNRs used for modification of thiol groups, such as NR-labeled derivatives of iodoacetamide and maleimide, yield strong covalent S-C bonds which do not permit release of the spin label from the protein. Chemical modification using a disulfide-containing SNRs permits subsequent demodification by a low-molecular weight thiol such as mercaptoethanol, reduced glutathione, cysteine or dithiothreitol. Such demodification, performed in conjunction with simultaneous measurements of activity and of structural characteristics, allows evaluation of the contribution of the group modified to the stability and 3D structure of the protein studied. Berliner et al. (8) used the spin label MTSSL, for reversible chemical

modification of Cys 25 in papain. We made use of biradical ·**RS-SR**· for the reversible chemical modification of NADPH-cytochrome P-450 reductase from rat liver (6,34), human hemoglobin (7), *Torpedo californica* acetylcholinesterase (*Tc*AChE) (35-37), TBADH (10,21) and allinase from garlic (27).

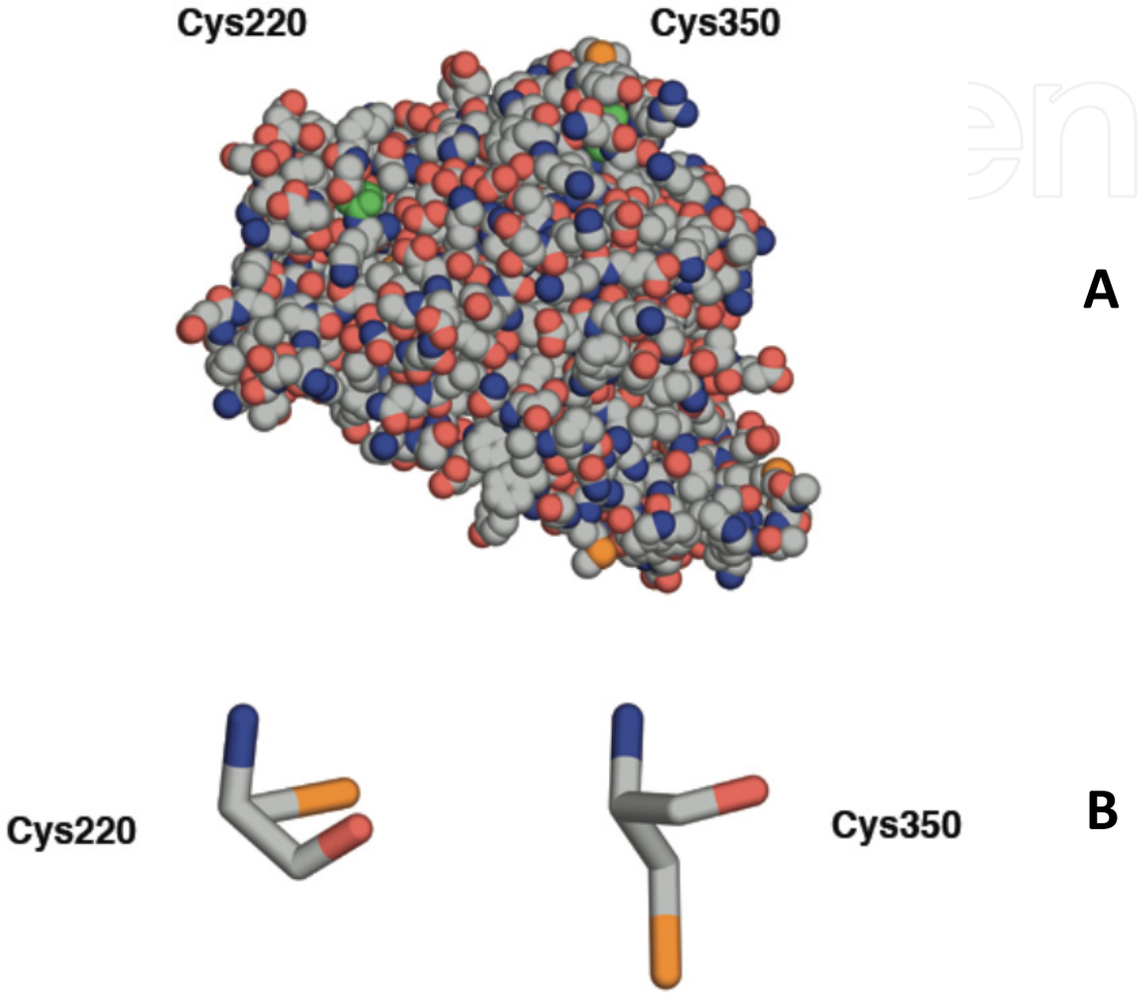
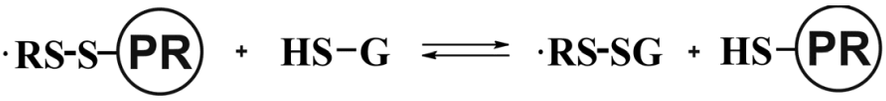


Figure 8. Free thiols of alliinase. (**A**) Relative locations of Cys220 and Cys350 (green) on the surface of the alliinase monomer. (**B**) Respective orientations of CysS220 and Cys350 relative to A. (from ref. 27)

4.1. Acetylcholinesterase from *Torpedo Californica* (*Tc*AChE)

Cys 231, a deeply buried residue in *Tc*AChE, was modified by biradical (eq.4) to yield a catalytically inactive species, even though it is not involved in the active site of the enzyme, which is a serine hydrolase (35-37).

(4)



(5)

Demodification of spin labeled protein by GSH (see eq.5), with concomitant release of the free monoradical spin label, done by ESR control, did not result in recovery of enzymatic activity. The use of a wide repertoire of physicochemical and biochemical techniques subsequently established that both the modified and demodified enzymes had assumed a partially unfolded, molten globule, **MG**, conformation (38,39). In such cases, where the chemical modification induced unfolding of the protein, there was a concomitant complete disappearance of ellipticity in the near-UV of the CD spectrum (λ_{min} = 280 nm), red shift of the maximum of intrinsic fluorescence spectrum (**333 nm → 341 nm**) and enzyme inactivation. However, changes of the secondary structure were very modest (35). Chemical modification Cys 231 by organo-mercurials nitroxyl radical: (2,2,5,5-Tetramethyl-4-[2-(chloromercuri)phenyl]-3-imidazoline-1-oxy1 (**HgR·**) and the natural thiosulfinate, allicin (see sheme 1), transforms *Tc*AChE to a quasi-native (**N***) state (36,40). Note, that modification by **HgR·** and allicin was also reversible and modified AChE was demodified by treatment with reduced glutathione (eq 5). Demodification of the organomercurial (or allicin) modified enzyme with GSH shortly after modification leads to regeneration of the physicochemical characteristics of the native enzyme as well as to *Tc*AChE reactivation. The modified enzyme in **N*** state is, however, metastable, and is converted spontaneously and irreversibly, at room temperature, with $t_{1/2} \simeq$ 100 min, to an **MG** state. Using the developed approach we were able to describe the conformational states of *Tc*AChE, transitions between these states (**N**- native, **U**-unfolded state), stimulation transition to MG state by biological membranes (41,42) as well as stabilization of **N** and **N*** states by chemical and pharmacological chaperons (40,43)

N ⇔ N* ⇨ MG ⇔ U

Scheme 2.

4.2. TBADH and NADPH-cytochrome P-450 reductase

Chemical modification of both TBADH and NADPH-cytochrome P-450 reductase by biradical ·**RS-SR**· (6,10,21,34)) also led to their inactivation (see Fig. 9)

However, in both cases removal of the bound spin label by treatment with the free thiol according to eq,5, resulted in immediate reactivation (Fig. 9). Spectroscopic measurements showed that modification had changed neither the tertiary nor secondary structure of the proteins and could be protected by affine inhibitor $NADP^+$.

4.3. Alliinase from garlic (*Allium sativum*)

We have shown recently that modification of Cys 220 and Cys 350 of alliinase with ·**RS-SR**· does not change its enzymatic activity (27). In this case chemical modification of both free cysteine residues was found to leave both the secondary and the tertiary structure of the enzyme unchanged. This might be attributable to the marked thermodynamic and structural

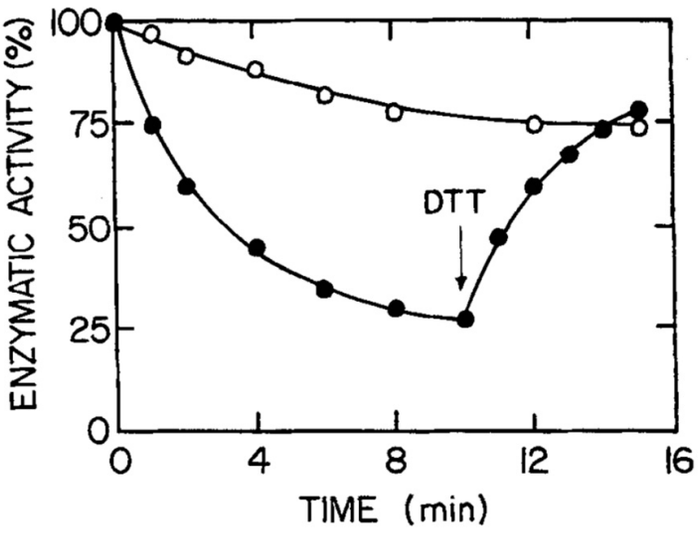


Figure 9. Influence of $NADP^+$ and DTT on effect of biradical ·**RS-SR**· on TBADH activity. Enzyme was incubated with the biradical without (closed circles) and with (open circles) $NADP^+$. DTT was introduced at 10 min (shown by arrow). (from ref. 21)

stability of alliinase, as well as the relatively long distances from modified free cysteines to the active center of the enzyme (see fig 7). This experimental finding permits one to use cysteines of alliinase for covalent binding with antibodies for targeted delivery of enzyme and for site-specific allicin generation to inhibit cancer cells proliferation (44,45).

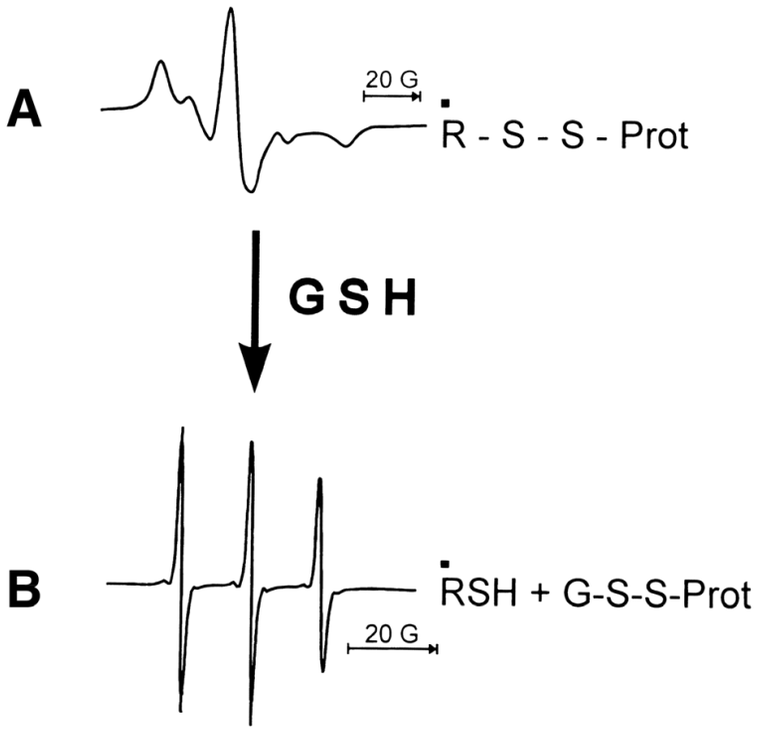


Figure 10. ESR spectrum of Alliinase-radical conjugate. Protein concentration was 13 μM in 10 mM PBS buffer, pH7.6. **(A) -** ESR spectrum of modified protein, ESR conditions: microwave power 10 mW; modulation amplitude 1 G; gain $2x10^5$. **(B)**- ESR spectrum of sample **(A)** 4 min after addition of 0.2 mM of glutathione. ESR conditions were the same as in **(A)**, but the gain was $3.2x10^4$.

5. Conclusions

For quantitate determination of sulfhydryl groups in low molecular weight compounds and proteins the symmetrical biradical containing disulfide bond, bis(2,2,5,5-tetramethyl-3-imidazoline-1-oxyl-4-il)-disulfide, or ·**RS-SR**· , was synthesized. The biradical, ·**RS-SR**· permits quantitative determination of glutathione and cysteine in biological systems ('thiol status') by use of ESR. Note that any *routine* CW ESR spectrometer can be used for the measurements! This non-invasive express method has very high sensitivity: possibility of measuring < 10^{-12} mol of SH groups in a sample. Thus, concentrations of glutathione and cysteine in living cells were determined under physiological conditions, as well as in cells, including cancer cells, treated by pro-and anti-oxidants.

Different from the traditional spin label method, the ·**RS-SR**· usage makes it possible to assess the rate of modification of SH groups in proteins (availability) and the influence of substrates, inhibitors, coenzymes, other proteins, artificial and biological membranes, pH, etc. in direct experiments. Modification of SH groups in proteins by ·**RS-SR**· is *reversible*, which permits application of this approach, coupled with site directed spin labeling, for evaluation of stability and unfolding of proteins and different parts of the protein globule.

Author details

Lev Weiner
Weizmann Institute of Science, Rehovot, Israel

6. References

[1] Kim, P.S. and Baldwin, R.L.(1990) Intermediates in the folding reactions of small proteins. Ann.Rev.Biochem. 59, 631-660

[2] Freedman, R.B. (1995) The formation of protein disulphide bonds. Curr. Opin. Struct. Biol. 5, 85-91

[3] Eichler, J., Kreimer, D.I., Varon, L., Silman, I., and Weiner, L. (1994) A 'molten globule' of Torpedo acetylcholinesterase undergoes thiol-disulfide exchange. J. Biol. Chem., 269, 30093-30096

[4] Methods in Enzymology: Biothiols,v.251 A,B (Packer, L.,Ed.) 1995.

[5] Ellman G.L. (1959) Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82 , 70–77

[6] Elinova, V.I., Slepneva, I.A., Weiner, L.M. Affinity modification of NADPH-Cytochrome P450 Reductase by reversible inhibitors.In: Cytochrome P-450, Biochemistry and Biophysics (I. Shuster, ed.) pp.411-414, 6th International Conference Vienna, Austria 1988., London, Taylor and Francis,1989.

[7] Khramtsov, V.V., Elinova, V.I., Weiner, L.M., Berezina, T.A. Martin, V.V., and Volodarsky, L.B. (1989) Quantitative determination of SH groups in low- and high-molecular-weight compounds by an electron spin resonance method. Anal. Biochem., 182, 58-63

[8] Berliner,LJ., Grunwald,J., Hankovszky, O. and Hideg, K., (1982) A novel reversible thiol-specific spin label: papain active site labeling and inhibition. Anal. Biochem. 119, 450-455

[9] Luckhurst, G.R. Biradicals as Spin Probes. In: Spin Labeling, Theory and Applications, (L.J. Berlinrer, ed) p.113, Academic Press, New York, (1976)

[10] Weiner, L.M. (1995) Quantitative determination of thiol groups in low and high molecular weight compounds by electron paramagnetic resonance. Methods in Enzymology, 251, 87-105

[11] Nohl, H., Stolze, K., and Weiner, L.M. (1995) Noninvasive measurement of thiol levels in cells and isolated organs. Methods in Enzymology 251, 191-203

[12] Khramtsov VV, and Volodarsky LB. Use of imidazoline nitroxides in studies of chemical reactions: ESR measurements of concentration and reactivity of protons, thiols and nitric oxide. In: Biological Magnetic Resonance (Berliner LJ, editor). Vol. 14. Spin labelling: the next millennium New York: Plenum Press; 1998, pp. 109–179

[13] Weiner, L.M. (2007 Stable nitroxyl radicals as pH, thiol and electron transfer probes Appl. Magn. Res. 31, 357-373

[14] Weiner, L.M., Hu, H., and Swartz, H. (1991) EPR method for the measurement of cellular sulfhydryl groups. FEBS Letters, 290, 243-246

[15] Pinkus, R., Weiner, L., and Daniel, V. (1995) Role of quinone-mediated generation of hydroxyl radicals in the induction of glutathione S-transferase gene expression. Biochemistry 34, 81-88.

[16] Pinkus, R., Weiner, L., and Daniel, V. (1996) Role of oxidants and antioxidants in the induction of AP-1, NF-kappa B, and glutathione S-transferase gene expression J. Biol. Chem. 271, 13422-13429

[17] Hirsch K, Danilenko, M, Giat J, Miron, T, Rabinkov, A, Wilchek, M, Mirelman, D, Levy, J, and Sharoni, Y. (2000) Effect of purified allicin, the major ingredient of freshly crushed garlic, on cancer cell proliferation. Nutrition and Cancer, 38, 245-254

[18] Khramtsov, V.V., Gorunova, T.E., and Weiner, L.M.: (1991) Monitoring of enzymatic activity *in situ* by EPR Biochem. Biophys. Res. Commun., 179, 520-527

[19] Ionita,P., Caragheorgheopol,A., Gilbert,B.C., and Chechik.V. (2004) Mechanistic Study of a Place-Exchange Reaction of Au Nanoparticles with Spin-Labeled Disulfides. Langmuir 20, 11536-11544

[20] Roshchupkina GI, Bobko AA, Bratasz A, Reznikov VA, Kuppusamy P, and Khramtsov VV. (2008) *In vivo* EPR measurement of glutathione in tumor-bearing mice using improved disulfide biradical Free Radic Biol Med. 45,312-330

[21] Peretz, M., Weiner, L., and Burstein, Y. Protein Science (1997) Cysteine reactivity in *Thermoanaerobacter brockii* alcohol dehydrogenase 6, 1074-1083

[22] Korkhin,Y, Kalb(Gilboa), A. J., Peretz, M., Bogin, O., Burstein, Y., and Frolow,F. (1998) NADP-dependent bacterial alcohol dehydrogenases: crystal structure, cofactor binding and cofactor specificity of the ADHs of *Clostridium beijerinckii* and *Thermoanaerobacter brockii*. J. Mol. Biol. 278, 965-979

[23] Rahman, M.S. (2007) Allicin and other functional active components in garlic: Health benefits and bioavailability. Int. J. Food Properties 10,245–268

[24] Rabinkov A, Miron T, Konstantinovski L, Wilchek M, Mirelman D, and Weiner, L.(1998) The mode of action of Allicin: Trapping of radicals and interaction with thiol containing proteins. Biochim Biophys Acta 1379, 233–244

[25] Kuettner EB, Hilgenfeld R, Weiss MS.(2002) The active principle of garlic at atomic resolution. J Biol Chem 277, 46402–46407

[26] Shimon LJ, Rabinkov A, Shin I, Miron T, Mirelman D, Wilchek M, and Frolow F.(2007) Two structures of alliinase from Alliium sativum L.: apo form and ternary complex with aminoacrylate reaction intermediate covalently bound to the PLP cofactor. J. Mol. Biol. 366,611–625

[27] Weiner, L., Shin, I; Shimon, LJW., Miron, T., Wilchek, M., Mirelman, D., Frolow, F. and Rabinkov. A. (2009) Thiol-disulfide organization in alliin lyase (alliinase) from garlic (*Allium sativum*). Protein Science 18, 196-205

[28] Kokorin AI, Zamaraev KI, Grigorian GL, Ivanov VP, and Rozantsev EG., (1972) Measurement of the distance between paramagnetic centers in solid solutions of nitrosyl radicals, biradicals and spin-labelled proteins. Biofozika, 17,34-39

[29] Mevorat-Kaplan K., Weiner, L. and Sheves M., J. Phys. Chem. B. (2006) Spin labeling of *Natronomonas pharaonis halorhodopsin*: probing the cysteine residues environment. J. Phys. Chem B, 110, 8825-8831

[30] Schiemann, O. and Prisner,T. (2007) Applications of electron paramagnetic resonance to distance measurements in biomolecules. Quarterly Reviews of Biophysics; 40, 1-53

[31] Altenbach,C., Marti, T., Khorana,H.G., and Hubbell, W.L.(1990) Transmembrane protein structure: spin labeling of bacteriorhodopsin mutants. Science, 248, 1088-1092

[32] Hubbell, W.L. and Altenbach,C. (1994) Investigation of structure and dynamics in membrane proteins using site-directed spin labeling. Curr.Opin. Struct.Biol., 4, 566- 573

[33] Eliash, T., Weiner,L., Ottolenghi, M., and Sheves,M.(2001) Specific binding sites for cations in bacteriorhodopsin. Biophys. J. 81, 1155-1162

[34] Elinova, V.I., Weiner, L.M., Slepneva, I.A., and Levina, A.S. (1993) Reversible Modification of Cysteine Residues of NADPH-Cytochrome P450 Reductase. Biochem. Biophys. Res. Commun. 193, 1044-1048

[35] Dolginova, E., Roth, E., Silman, I., Weiner, L.(1992) Chemical modification of *Torpedo* acetylcholinesterase by disulfides: Appearance of a "molten globule" state . Biochemistry, 31, 12248-12254

[36] Kreimer, D., Dolginova, E., Raves, M., Sussman, J., Silman, I., and Weiner, L.M. (1994) A metastable state of *Torpedo californica* acetylcholinesterase generated by modification with organomercurials.Biochemistry, 33, 14407-14418

[37] Kreimer, D.I., Shin, I., Shnyrov, V.L., Villar, E., Silman, I., and Weiner, L.(1996) Two partially unfolded states of *Torpedo californica* acetylcholinesterase. Protein Sci. 5,1852-1864

[38] Arai, M. and Kuwajima, K.:(2000) Role of the molten globule state in protein folding . Adv. Protein Chem. 53, 209-282

[39] Englander, S.W. (2000) Protein folding intermediates and pathways studied by hydrogen exchange. Annu Rev. Biophys. Biomol. Struct. 29, 213-238

[40] Millard, CB., Shnyrov, VL.,Newstead, S., Shin, I., Roth, E., Silman, I and Weiner, L.(2003) Stabilization of a metastable state of *Torpedo californica* acetylcholinesterase by chemical chaperones. Protein Sci. 12, 2337-2347,

[41] Shin, I., Kreimer, D.I., Silman, I. & Weiner, L. (1997). Membrane-promoted unfolding of acetylcholinesterase: A possible mechanism for insertion into the lipid bilayer. Proc. Natl. Acad. Sci. USA, 94, 2848-2852

[42] Shin, I., Silman,I., Bon, C., and Weiner ,L. (1998) Liposome-catalyzed unfolding of acetylcholinesterase from Bungarus fasciatus. Biochemistry, 37, 4710-4716

[43] Weiner, L., Shnyrov, VL., Konstantinovskii, L., Roth, E., Ashani, Y.,and Silman,I. (2009) Stabilization of *Torpedo Californica* Acetylcholinesterase by Reversible Inhibitors. Biochemistry 48, 563-574

[44] Appel, E ., Vallon-Eberhard, A., ; Rabinkov, A .,Brenner, O., Shin, I., Sasson, K., Osherov, N., Jung, S., and Mirelman, D. Antimicrob. Agents Chemotherap. 54, 898-906 (2010)

[45] Appel, E ., Rabinkov, A., Neeman, M., Kohen, F., and. Mirelman, D.(2011) Conjugates of daidzein-alliinase as a targeted pro-drug enzyme system against ovarian carcinoma. J. Drug. Target. 19, 326-335