Diastereoselective reduction of protein-bound methionine sulfoxide by methionine sulfoxide reductase

Victor S. Sharov[a], Deborah A. Ferrington[b], Thomas C. Squier[b], Christian Schöneich[a,*]

[a] *Department of Pharmaceutical Chemistry, University of Kansas, 2095 Constant Avenue, Lawrence, KS 66047, USA*
[b] *Department of Molecular Biosciences, University of Kansas, Lawrence, KS 66045, USA*

Received 14 June 1999; received in revised form 21 June 1999

Abstract **Methionine sulfoxide (MetSO) in calmodulin (CaM) was previously shown to be a substrate for bovine liver peptide methionine sulfoxide reductase (pMSR, EC 1.8.4.6), which can partially recover protein structure and function of oxidized CaM in vitro. Here, we report for the first time that pMSR selectively reduces the D-sulfoxide diastereomer of CaM-bound L-MetSO (L-Met-D-SO). After exhaustive reduction by pMSR, the ratio of L-Met-D-SO to L-Met-L-SO decreased to about 1:25 for hydrogen peroxide-oxidized CaM, and to about 1:10 for free MetSO. The accumulation of MetSO upon oxidative stress and aging in vivo may be related to incomplete, diastereoselective, repair by pMSR.**

Key words: Calmodulin; Oxidative stress; Hydrogen peroxide; Methionine sulfoxide; Peptide methionine sulfoxide reductase; Diastereoselectivity

1. Introduction

The oxidation of methionine (Met) to methionine sulfoxide (MetSO) represents a common post-translational protein modification accompanying biological conditions of oxidative stress [1–5] and aging [6–9]. Depending on the location of the modified Met residue, MetSO formation may cause conformational changes and/or inactivation of a protein [1,5,10], and has been implicated in the modulation of cellular signaling processes [6,7,11]. Recently, it has been suggested that the oxidation of surface-exposed Met residues to MetSO may represent an endogenous antioxidant defense that protects proteins against the oxidation of other critically important residues [3]. An essential part of all these hypotheses is the fact that these MetSO residues can be reduced back to Met by the enzyme peptide methionine sulfoxide reductase (pMSR) [3–6,11–13].

Oxidation of L-Met theoretically generates two MetSO diastereomers, L-Met-D-SO and L-Met-L-SO. Although no data are currently available regarding the MetSO diastereomer composition in biological systems, our recent in vitro results show that Met oxidation through biologically relevant reactions, involving Met radical cations and superoxide, can be diastereoselective [14]. Moreover, the enzymatic reduction of MetSO by pMSR has been suggested to be diastereoselective; incubation of 4-dimethylamino-azobenzene-4′-sulfonyl L-Met-SO (DABS-L-MetSO) with a crude protein extract from human polymorphonuclear leukocytes has been reported to reduce DABS-L-Met-L-SO about 10 times faster than DABS-L-Met-D-SO [15].

In contrast, here we show that purified bovine liver pMSR is diastereoselective towards L-Met-D-SO both for free and protein-bound MetSO, present in oxidized calmodulin (CaM). Calmodulin was chosen for this study as our recent results show that CaM accumulates a significant fraction of MetSO in vivo during biological aging [7,8].

2. Materials and methods

2.1. Reagents

TPCK-treated sequence grade trypsin was from Promega (Madison, WI); physiological amino acid standards, and carboxypeptidase Y from baker's yeast were from Pierce (Rockford, IL). γ-Methylsulfinyl-α-aminobutyric acid (L-methionine-DL-sulfoxide, MetSO), *o*-phthalaldehyde (OPA), NADPH, catalase, thioredoxin reductase from *Escherichia coli*, *n*-amylamine and sodium acetate were from Sigma (St. Louis, MO). Glutathione Sepharose 4B was from Pharmacia Biotech (Uppsala, Sweden), and phenyl Sepharose CL-4B was from Pharmacia (Piscataway, NJ). Thioredoxin from *Spirulina* sp. was from Fluka Biochemika (Buchs, Switzerland). Picric acid was from Aldrich (Milwaukee, WI). All other chemicals of the highest commercially available grade were from Fisher (Pittsburgh, PA).

2.2. Expression and purification of recombinant proteins

CaM was prepared essentially as previously described [10]. A single isoform of CaM corresponding to the c-DNA encoding vertebrate CaM provided by Prof. Sam George (Duke University) was subcloned into the expression vector pALTER-Ex1 (Promega, Madison, WI), over-expressed in *E. coli* strain JM109 (Promega, Madison, WI), and purified by weak anion exchange HPLC using phenyl Sepharose CL-4B [10,16]. The sequence of CaM, in the one-letter code (methionine residues are typed in bold), with trypsin cleavage fragments T_1–T_{14} divided by arrows, is presented below:

T_1 T_2 T_3 T_4 T_5
ADQLTEEQIAEFK↓EAFSLFDK↓DGNGTITTK↓ELGTV**M**R↓SLGQNPTEAELQD
T_5 T_6 T_7 T_8 T_9 T_{10} T_{11}
MINEVDADGNGTIDFPEFLT**MM**AR↓K↓**M**K↓DTDSEEEIR↓EAFR↓FVDK↓DGNG
T_{11} T_{12} T_{13} T_{14}
YISAAELR↓HV**M**TNLGEK↓LTDEEVDE**M**IR↓EANIDGDGEVNYEEFVQ**MM**TAK

Bovine liver pMSR was expressed in *E. coli* as a GST fusion protein, and purified using a glutathione Sepharose 4B affinity column as described [13]. Proteins were aliquoted and stored at −70°C.

2.3. Oxidation of calmodulin

CaM (40 μM) was oxidized at 25°C for 3 h in air-saturated buffer containing 20 mM sodium phosphate, pH 7.4, containing 100 mM KCl and 5 mM H_2O_2. The reaction was stopped by the addition of 10 U/ml catalase. The oxidized protein was rapidly separated from the other reactants on a Vydac C4 guard column (4.6×25 mm), initially equilibrated with aqueous trifluoroacetic acid (TFA; 0.1%), by elution with 80% MeOH/20% H_2O/0.1% TFA (v/v). CaM samples were collected, dried under vacuum, and stored at −20°C.

2.4. Tryptic digestion of CaM and RP HPLC analysis of the digest

Exhaustive tryptic digestion of CaM was performed at 37°C in

*Corresponding author. Fax: (1) (785)-864-5736.
E-mail: schoneich@hbc.ukans.edu

PII: S0014-5793(99)00888-1

50 mM potassium phosphate buffer, pH 8.0, at a molar ratio of CaM to trypsin of 60:1. The reaction was terminated after 12 h by the addition of TFA, adjusting the solution to pH 2. Aliquots of 100 μl were separated by HPLC (Varian 9012) on a Vydac C4 column (4.6×250 mm, equipped with a 4.6×25 mm guard column), equilibrated with 0.1% aqueous TFA, and eluted at 1 ml/min with a linear gradient increasing the acetonitrile content to 45% in 0.1% TFA at a rate of 0.75%/min. Tryptic fragments were monitored at 215 nm by UV absorbance detector (Varian 9050). Peaks of interest were collected and lyophilized. Identification of all the tryptic fragments of native and oxidized CaM was carried out by electro spray ionization-mass spectrometry (ESI MS) as described [17].

2.5. Carboxypeptidase Y digestion

The MetSO containing tryptic fragment T_{14}(O) (~3 μM), collected after HPLC separation of an exhaustive tryptic digest of oxidized CaM was incubated for 1 h at 25°C with carboxypeptidase Y (1 U/ml) in 10 mM sodium phosphate buffer, pH 7.0, containing 0.1 mM EDTA. The reaction was stopped by mixing with an equal volume of 0.1 M acetic acid, adjusting the final pH to about 3.0.

2.6. Reduction of methionine sulfoxide in oxidized calmodulin or free methionine sulfoxide with peptide methionine sulfoxide reductase

Oxidized CaM (40 μM) or 70 μM L-Met-DL-SO were incubated for 2 h at 37°C in the presence of various concentrations of pMSR and thioredoxin/thioredoxin reductase/NADPH (T/TR/NADPH) [13] in 20 mM sodium phosphate, pH 7.4, containing 100 mM KCl. Incubation with T/TR/NADPH alone served as a control for the effect of pMSR.

2.7. HPLC analysis of L-MetSO diastereomers

Sulfoxide diastereomers were separated by HPLC after pre-column OPA derivatization. The derivatization reagent was freshly prepared by mixing equal volumes of an OPA stock solution (containing 25 mg OPA, 0.625 ml methanol, 25 μl 2-mercaptoethanol, and 11.2 ml 0.4 M K_2HPO_4 buffer, adjusted to pH 9.5 with sulfuric acid) with 1% Brij in the same buffer. Sample solutions (10 μl) were mixed at room temperature with 40 μl of the OPA/Brij derivatization reagent and after exactly 1 min, 80 μl of 0.4 M KH_2PO_4 were added. After a further minute, 100 μl of the mixture were injected onto a Zorbax C18-Rx column (4.6×150 mm) initially equilibrated at 35°C with 20 mM sodium acetate, pH 5.8 (mobile phase A). At the time of injection the mobile phase was switched to 90% A/10% methanol and eluted isocratically during 38 min at 2 ml/min, followed by a washing step at 10% A/90% methanol for 10 min. Amino acid derivatives were monitored by flow fluorescence detector (Shimadzu RF-10AXL) with excitation and emission wavelengths set at 330 and 450 nm, respectively. Calibration was performed using racemic L-MetSO (Sigma). Control experiments (data not presented) showed that the ratio of 1.13±0.04 for the OPA-derivatives of L-Met-D-SO and L-Met-L-SO, obtained for a standard MetSO racemic mixture (Table 1) differs slightly from a theoretical value of unity due to the instability of the OPA-derivatives (half-life estimated at 35°C was about 60 min) and a difference in retention time of the diastereomers of about 2 min.

2.8. Preparation of authentic standards

Authentic standards of L-Met-D-SO and L-Met-L-SO were prepared according to the method of Lavine [18] with minor modifications. Briefly, 0.3 g of L-methionine-DL-sulfoxide were dissolved in 2 ml H_2O in a boiling water bath. After slight cooling, a methanolic solution of picric acid (0.5 g wet weight/4 ml) was slowly added to give a yellow precipitate. After cooling to room temperature, the precipitate was filtered off through cellulose paper, washed with ice-cold methanol, and re-dissolved in 20 ml H_2O. To liberate the sulfoxide, amylamine (80 μl) was added, bringing the final pH to around 8.0. L-Met-D-SO was reprecipitated by the addition of 200 ml acetone, and collected by centrifugation (12 000×g for 5 min). The pellet was washed twice with acetone and dried under vacuum. The product had a specific rotation of $[\alpha]_D^{25} = +100°$ in H_2O, identical to the published value for L-Met-D-SO [18], and its OPA derivative coeluted (>98% purity) with peak 'd' shown in Fig. 1A.

The original filtrate was evaporated under vacuum to remove methanol, redissolved in 20 ml H_2O+80 μl amylamine, and 200 ml acetone were added to reprecipitate the free sulfoxide. The pellet was collected by centrifugation, washed twice with acetone and dried. After OPA derivatization the resulting sulfoxide was found to consist of 75% L-Met-L-SO and 25% L-Met-D-SO.

3. Results

3.1. Oxidation of methionine residues in calmodulin to methionine sulfoxide

Incubation of CaM with H_2O_2 led to oxidation of Met predominantly in the C-terminal fragment T_{14} (Glu^{127}–Lys^{148}; containing Met^{144} and Met^{145}) to the overall mono-oxidized fragment T_{14}(O), as assessed by tryptic mapping and ESI-MS analysis. Relatively mild conditions ensured the conversion of 23.0±1.8% of either Met^{144} or Met^{145} to MetSO (Table 1), whereas other Met-containing fragments displayed at least three-fold lower oxidization yields (data not presented).

3.2. Diastereoselectivity of methionine sulfoxide reduction in CaM by peptide methionine sulfoxide reductase

Subsequent to the in vitro oxidation by H_2O_2, CaM was incubated with pMSR followed by trypsin digestion. The oxidized tryptic fragment, T_{14}(O), was collected and subjected to digestion by carboxypeptidase Y. Fig. 1A–C demonstrate that pMSR selectively targets the CaM-bound L-Met-D-SO. Both diastereomers, L-Met-D-SO and L-Met-L-SO are well separated in an amino acid standard mixture (Fig. 1A), present in the carboxypeptidase Y digest of T_{14}(O) of H_2O_2-treated CaM (Fig. 1B), but L-Met-D-SO disappeared after the exposure of oxidized CaM to pMSR (Fig. 1C). Table 1 shows that the selective reduction of L-Met-D-SO requires the presence of pMSR, and depends on the concentration of pMSR. The T/TR/NADPH system alone was almost ineffective at MetSO

Table 1
MetSO diastereomer ratio in T_{14} tryptic fragment of CaM after oxidation of the protein with H_2O_2 and following incubation with pmsR

Experimental conditions	% of MetSO in T_{14}[a]	D/L ratio[b]
L-Methionine-DL-sulfoxide (racemic)	–	1.13±0.04
Native CaM	1.8±0.3	–
Oxidized CaM (CaM_{ox})[c]	23.0±1.8	1.13±0.04
CaM_{ox}+T/TR/NADPH[d]	20.8±2.5	1.16±0.05
CaM_{ox}+T/TR/NADPH[e]+0.25 μM pMSR	13.3±2.4	0.40±0.05
CaM_{ox}+T/TR/NADPH[e]+0.5 μM pMSR	10.3±0.3	0.038±0.006

[a]Determined by the HPLC and ESI-MS in CaM tryptic digest.
[b]Determined by the HPLC with pre-column OPA derivatization in carboxypeptidase Y digest of T_{14}(O) CaM tryptic fragment.
[c]CaM (40 μM) was incubated at 25°C for 3 h with 5 mM H_2O_2 in air-saturated buffer containing 100 mM KCl, 20 mM sodium phosphate, pH 7.4.
[d]Oxidized CaM (40 μM) was incubated at 37°C for 2 h with 1 μM T, 0.5 μM TR, and 500 μM NADPH.
[e]At molar ratios of T/TR/NADPH to pMSR of 2:1, 1:1 and 500:1, respectively.

reduction even at a maximal concentration used, and did not change the ratio of L-Met-D-SO/L-Met-L-SO.

3.3. Diastereoselectivity of pMSR towards free methionine sulfoxide

In order to assess whether the observed diastereoselectivity was dependent on L-MetSO bound to a protein, we subjected a D/L mixture of the free amino acid, L-Met-DL-SO to reduction by pMSR. Incubation of free L-Met-DL-SO with pMSR resulted in a preferential reduction of L-Met-D-SO (Fig. 2). The L-sulfoxide remained nearly intact unless a very high concentration of pMSR was used, so that the D/L ratio decreased to about 1:10 at a maximal pMSR concentration (Fig. 2). Thus, pMSR shows the same diastereoselectivity towards free and peptide-bound MetSO.

4. Discussion

Our report shows for the first time that pMSR diastereoselectively reduces protein-bound L-Met-D-SO. In fact, the D/L ratio of the remaining MetSO was significantly less after the reduction of protein-bound L-MetSO (~1:25) as compared to free L-MetSO (~1:10)[1]. The high preference for L-Met-D-SO is in contrast to the work of Minetti et al. [15]. However, source and purity of the enzymes were different in both studies: whereas Minetti et al. used a crude extract from human polymorphonuclear leukocytes, we utilized purified bovine liver pMSR. Furthermore, our results were obtained with physiological reductants, T/TR/NADPH, as compared to DTT and DTE in [15], using physiological substrates for pMSR. Differences in the diastereoselectivity of MetSO reduction may also indicate the existence of more than one enzymatic system reducing MetSO diastereomers, depending on the nature of the tissue and conditions, e.g. oxidative stress at inflammation sites where activated leukocytes are implicated.

Considering the potential antioxidant function of the pMSR/Met system, it is rather difficult to understand such a high selectivity of a repair enzyme towards only one sulfoxide diastereomer. In vitro studies showed that the oxidation of free L-Met by chloroperoxidase [19] as well as by hydrogen peroxide (Table 1) or peroxynitrite (our unpublished data) produces 1:1 ratios of L-Met-D-SO and L-Met-L-SO, although a partial asymmetric oxidation of Met (ultimately giving higher yields of D-sulfoxide) was shown in model reactions involving Met radical cations and superoxide [14]. Pending a detailed analysis of the diastereomer ratios of L-Met-D-SO and L-Met-L-SO in oxidized proteins in vivo, we can expect the repair of MetSO in vivo to be incomplete. This may be one rationale for the accumulation of MetSO on CaM in senescent brain [7,8].

The stereospecificity of pMSR may invite other, though more speculative considerations. The antioxidant action of pMSR may merely be a side effect of an enzyme designed for performing a signaling function, based on a reversible diastereoselective protein Met oxidation. In this case a dia-

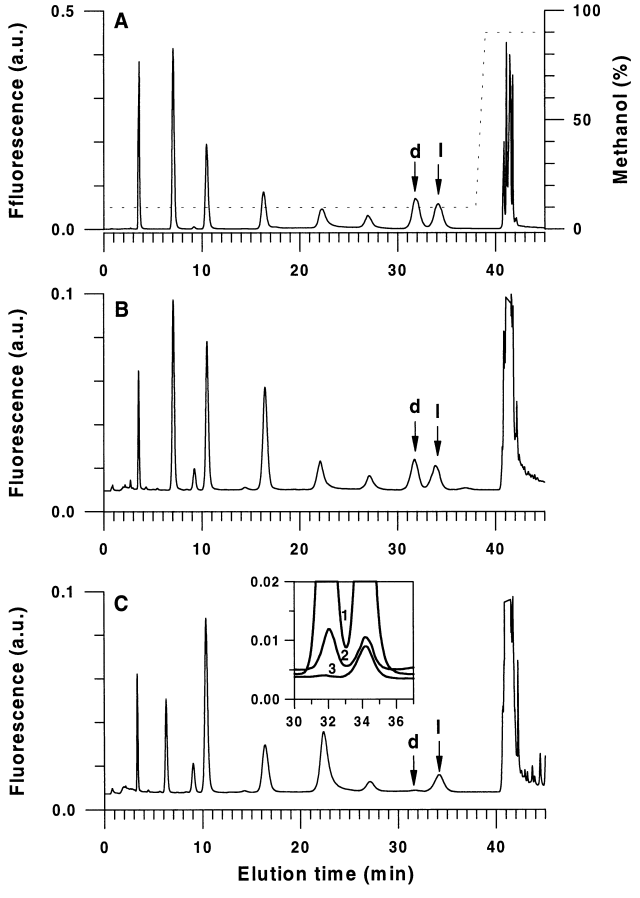

Fig. 1. Chromatograms of OPA-derivatized amino acids from (A) amino acid standards (Pierce, 12.5 μM each) +25 μM L-Met-DL-SO (Sigma), and from the carboxypeptidase Y digest of T_{14}(O) fragment for (B) oxidized CaM, and (C) oxidized CaM after the incubation for 2 h at 37°C with 0.5 μM pMSR, 1 μM thioredoxin, 0.5 μM thioredoxin reductase, and 250 μM NADPH. Dashed line in A shows the percent of organic modifier (methanol) in mobile phase (right axis). Arrows indicate a chromatographic peak position for D- and L-diastereomers of the MetSO OPA-derivatives. Inset in C: an overlay of the three extended chromatograms for L-MetSO peaks (1, A; 2, B; 3, C).

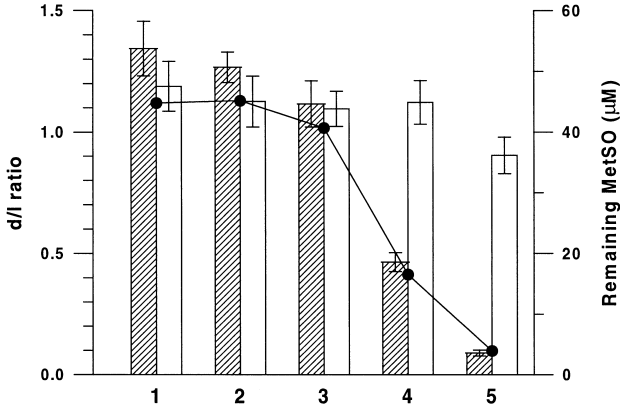

Fig. 2. Reduction of free L-methionine-DL-sulfoxide by pMSR. Shadowed columns: remaining L-Met-D-SO; open columns: L-Met-L-SO, line plot represents D/L diastereomeric ratio. Experimental conditions: 100 μM L-Met-DL-SO incubated for 2 h at 37°C either (1) alone or with (2) T/TR/NADPH of 3, 1.5, 750 μM, respectively; (3) T/TR/NADPH of 0.3, 0.15, 75 μM, respectively, and 0.15 μM pMSR; (4) T/TR/NADPH of 1.5, 0.75, 375 μM, respectively, and 0.75 μM pMSR; (5) T/TR/NADPH of 3, 1.5, 750 μM, respectively, and 1.5 μM pMSR.

[1] A similar diastereoselectivity for free L-Met-D-SO was also observed by Moskovitz et al. (J. Moskovitz, R.L. Levine and E.R. Stadtman, personal communication).

stereoselective MetSO reduction should be preceded by a diastereospecific, potentially enzymatic, MetSO formation. For example, an enzymatic mechanism for the oxidation of free Met with preferred formation of L-Met-D-SO was discovered in rabbit liver and kidney microsomes, characterized as a flavin-containing monooxygenase isozyme 3 [20,21].

Acknowledgements: This study was supported by the National Institutes of Health (Grant P01AG12993). We thank Todd D. Williams of the KU Mass Spectrometry Laboratory for acquiring the ESI MS spectra, and Drs. J. Moskovitz and R.L. Levine for providing the pMSR clone.

References

[1] Vogt, W. (1995) Free Radical Biol. Med. 18, 93–105.
[2] Hayes, C.S., Illades-Aguiar, B., Casillas-Martinez, L. and Setlow, P. (1998) J. Bacteriol. 180, 2694–2700.
[3] Levine, R.L., Mosoni, L., Berlett, B.S. and Stadtman, E.R. (1996) Proc. Natl. Acad. Sci. USA 93, 15036–15040.
[4] Moskovitz, J., Flescher, E., Berlett, B.S., Azare, J., Poston, J.M. and Stadtman, E.R. (1998) Proc. Natl. Acad. Sci. USA 95, 14071–14075.
[5] Sigalov, A.B. and Stern, L.J. (1998) FEBS Lett. 433, 196–200.
[6] Sun, H., Gao, J., Ferrington, D.A., Biesiada, H., Williams, T.D. and Squier, T.C. (1999) Biochemistry 38, 105–112.
[7] Gao, J., Yin, D.H., Yao, Y., Williams, T.D. and Squier, T.C. (1998) Biochemistry 37, 9536–9548.
[8] Michaelis, M.L., Bigelow, D.J., Schöneich, C., Williams, T.D., Ramonda, L., Yin, D., Hhhmer, A.F.R., Yao, Y., Gao, J. and Squier, T.C. (1996) Life Sci. 59, 405–412.
[9] Schöneich, C. (1999) Exp. Gerontol. 34, 19–34.
[10] Gao, J., Yin, D.H., Yao, Y., Sun, H., Qin, Z., Schöneich, C., Williams, T.D. and Squier, T.C. (1998) Biophys. J. 24, 1115–1134.
[11] Ciorba, M.A., Heinemann, S.H., Weissbach, H., Brot, N. and Hoshi, T. (1997) Proc. Natl. Acad. Sci. USA 94, 9932–9937.
[12] Moskovitz, J., Berlett, B.S., Poston, J.M. and Stadtman, E.R. (1999) Methods Enzymol. 300, 239–244.
[13] Moskovitz, J., Weissbach, H. and Brot, N. (1996) Proc. Natl. Acad. Sci. USA 93, 2095–2099.
[14] Miller, B.L., Kuczera, K. and Schöneich, C. (1998) J. Am. Chem. Soc. 120, 3345–3356.
[15] Minetti, G., Balduini, C. and Brovelli, A. (1994) Ital. J. Biochem. 43, 273–283.
[16] Strasburg, G.M., Hogan, M., Birmachu, W., Thomas, D.D. and Louis, C.F. (1988) J. Biol. Chem. 263, 542–548.
[17] Hühmer, A.F.R., Gerber, N.C., Ortiz de Montellano, P.R. and Schöneich, C. (1996) Chem. Res. Toxicol. 9, 484–491.
[18] Lavine, T.F. (1947) J. Biol. Chem. 169, 477–491.
[19] Ramakrishnan, K., Oppenhuizen, M.E., Saunders, S. and Fisher, J. (1983) Biochemistry 22, 3271–3277.
[20] Duescher, R.J., Lawton, M.P., Philpot, R.M. and Elfarra, A.A. (1994) J. Biol. Chem. 269, 17525–17530.
[21] Krause, R.J., Ripp, S.L., Sausen, P.J., Overby, L.H., Philpot, R.M. and Elfarra, A.A. (1996) Arch. Biochem. Biophys. 333, 109–116.