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Study of mammalian selenocysteyl-tRNA synthesis with [^{75}Se]HSe$^-$

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The mechanisms of the synthesis of mammalian selenocysteyl-(Scy)-tRNA were studied using [^{75}Se]H_2Se. H_2Se was prepared from [^{75}Se]selenite, glutathione, NADPH and glutathione reductase, and was purified by chromatography. It was confirmed that this H_2Se was a Se donor in the reaction of the synthesis of Scy-tRNA. [^{75}Se]Scy, liberated from aminoacyl-tRNA, was analyzed by TLC on silica gel an subsequent autoradiography. The activity of Scy-tRNA synthesis was found in the supernatant at 105 000 × *g* of the murine liver extract, but not in the precipitate. The supernatant was chromatographed on DEAE-cellulose, and the activity was eluted at a concentration of 0.17 M KCl. This position is at the front shoulder of the peak of seryl-tRNA synthetase which was eluted at 0.20 M KCl. Major serine $tRNA_{IGA}$ is not a substrate on which to synthesize Scy-tRNA, but natural opal suppressor serine tRNA is. On a chromatographic pattern of a Scy-tRNA preparation on Sephacryl S-200, the radioactivity of ^{75}Se was eluted at the tRNA peak. This showed that Scy bound to tRNA. The active protein fraction from DEAE-cellulose did not contain tRNA kinase, therefore Scy-tRNA must be directly synthesized from seryl-tRNA, not through phosphoseryl-tRNA. This mechanism is similar to that seen in *Escherichia coli* [1991, J. Biol. Chem. 266, 6324].

Selenocysteine, tRNA; Selenium, tRNA; tRNA, opal suppressor; tRNA, serine; tRNA, selenocysteine; selenocysteine synthase

1. INTRODUCTION

Selenocysteine (Scy) is contained in mammalian glutathione peroxidase (GSHPx) [1] and type-1 iodothyronine deiodinase [2]. Scy plays an important role at those active sites. The codon of Scy is UGA, which is normally used as an opal termination codon in protein synthesis [2,3]. The natural tRNA corresponding to UGA is called opal suppressor tRNA and accepts serine [4]. This tRNA is present in the cytosol as Scy-tRNA in vivo [5]. The carbon skeleton of Scy in GSHPx comes from serine [6]. Meanwhile, this suppressor seryl-tRNA is phosphorylated by tRNA kinase to become phosphoseryl(Ps)-tRNA [7]. It has been presumed that Scy in mammals is biosynthesized on seryl-tRNA by conversion of the serine moiety to an intermediate, Ps-tRNA [5,8,9]. However, the mechanisms in mammals are ambiguous because the activity of Scy-tRNA synthesis has not been clarified. Meanwhile, in *E. coli* the products of the *selA* and *selD* genes function in the biosynthesis of Scy-tRNA [10]. SELA catalyzes the conversion of seryl-tRNA into Scy-tRNA, and SELD is involved in selenium metabolism [10]. In order to clarify the mechanisms of the biosynthesis of mammalian Scy-tRNA, we searched for [^{75}Se]Scy-tRNA synthesis activity in murine liver extracts, and we show in this report that HSe^- (H_2Se is present as HSe^- in buffers at neutral pH) is a Se donor in the synthesis of Scy-tRNA.

Abbreviations: Scy, selenocysteine; Ps, phosphoserine; GSH, glutathione; GSHPx, glutathione peroxidase

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2. EXPERIMENTAL

Natural opal suppressor tRNA was prepared from bovine liver and chromatographed on BD-cellulose [11]. The last fraction eluted from BD-cellulose is rich in suppressor tRNA and separated from major serine tRNA. This fraction was used for the assay of a Scy-tRNA synthesis reaction. The serine acceptor activity of this fraction was a 20 pmol/A_{260} unit. Assays of the seryl-tRNA synthetase (SerRS) of molecular mass 65.5 kDa and tRNA kinase were performed according to the method in [12].

The enzyme of Scy-tRNA synthesis was prepared from murine liver of the ICR strain as follows. Liver was minced with quartz sand in five volumes of 0.25 M sucrose, 10 mM Tris-HCl, 10 mM mercaptoethanol at pH 7.5. This was centrifuged at 8000 × *g* for 10 min, and the supernatant was centrifuged at 105 000 × *g* for 60 min. The supernatant was chromatographed on a DEAE-cellulose column. Chromatographic conditions on DEAE-cellulose and Sephacryl S-300 were the same as in a previous report [12]. The activity of SerRS in each eluate was measured in a similar manner as in [12]. The activity of Scy synthase was measured by a method that will be described later. The active fraction of Scy synthase was dialyzed against 50% glycerol, 10 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM mercaptoethanol at pH 7.5, and the daily yield was stored at −80°C.

[^{75}Se]HSe^- was prepared from [^{75}Se]selenite by the enzymatic method of Ganther [13], as follows. [^{75}Se]Selenite (1 μl, 280 GBq/mmol, 370 MBq/ml) was mixed with 10 μl of 40 mM GSH, 1 μl of 10 mM NADPH, 5 μl of 0.2 M mercaptoethanol, 5 μl of water, 2 μl of 1 M HEPES-NaOH (pH 7.5), and 0.25 μl of yeast GSH reductase (a product of Boehringer). This mixture ('H_2Se mixture') was incubated overnight, and was used in preliminary experiments. For further experiments, HSe^- was purified from the above 'H_2Se mixture' by chromatography on Sephacryl S-200 in 10 mM mercaptoethanol, 10 mM acetate at pH 4.6. The radioactivity of [^{75}Se] in the eluates from the S-200 column was measured by a γ-autowell counter (Aloka ARC 301). Subsequently, eluates were analyzed by TLC as will be described

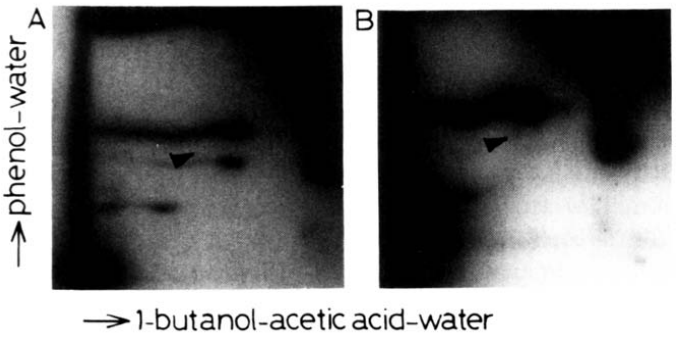

Fig. 1. Autoradiograph of ^{75}Se on TLC plates. The hydrolyzate of [^{75}Se]aminoacyl-tRNA was chromatographed on silica gel G. A, the enzyme preparation employed for Scy-tRNA synthesis was the precipitate at 105 000 × g of murine liver extracts; B, the enzyme was the supernatant at 105 000 × g. Arrows indicate the position of authentic Scy.

later. The fractions containing HSe^- were pooled and used for the reaction of the synthesis of Scy-tRNA. Authentic cold HSe^- was chemically prepared according to the method described in reference [14].

Scy synthase reaction was carried out as follows: suppressor tRNA (0.5 μmol) in 20 μl of 0.2 M HEPES at pH 7.4, 20 mM $MgCl_2$, 20 mM KCl, 20 mM mercaptoethanol was mixed with 1 μl of 40 mM serine, 5 μl of 50 mM ATP and 1 μl of SerRS. This was incubated for 20 min at 30°C. Afterwards, 10 μl (60 kBq) of 1.2 μM HSe^- and 10 μl of enzyme preparation for Scy-tRNA synthesis were added to the mixture and were further incubated for 2 h at 30°C. After the incubation, the tRNA was precipitated by an addition of ethanol and was collected. Part of this tRNA was chromatographed on Sephacryl S-200 in 0.15 M NaCl, 10 mM acetate, 10 mM mercaptoethanol (pH 4.6). The precipitate was washed with ethanol and then hydrolyzed in 5 μl of 1 M ammonium hydroxide solution in the presence of 1 M mercaptoethanol for 2 h at 30°C. Mercaptoethanol was added to protect the Scy from oxidation. After the hydrolysis, the tRNA was precipitated by the addition of ethanol, and the supernatant was immediately analyzed by TLC on silica gel G plates. Cold Scy (a generous gift of Prof. K. Soda of Kyoto University) was co-chromatographed. We confirmed that Scy was only slightly oxidized during spotting and development of the samples on the TLC plates. Cold Scy on the TLC plates was coloured by a ninhydrin reaction. The solvent system used for the TLC was as follows: first dimension, 1-butanol/acetic acid/H_2O (4:1:1); second dimension, phenol/H_2O (3:1, w/v) [1]. Occasionally, the activity in many enzyme fractions was measured by the analysis of hydrolyzates on one-dimensional TLC (solvent: 1-butanol/acetic acid/H_2O). The radioactivity of ^{75}Se on TLC was clarified by standard autoradiography. For quantitative measurement of radioactivity of ^{75}Se, silica on TLC plates was scraped and the radioactivity on the silica was determined by a γ-autowell counter.

3. RESULTS

To search for the activity of Scy-tRNA synthesis, we first used the 'H_2Se mixture' by Ganther [13], as a Se donor. The mixture was composed of selenite, GSH, NADPH and GSH reductase. The analysis of the tRNA product revealed a weak spot of Scy when a supernatant at 8000 × g was used (data not shown). This supernatant at 8000 × g was centrifuged at 105 000 × g, and the activity was found in the supernatant at 105 000 × g, as shown in Fig. 1B. The precipitate at 105 000 × g did not contain any activity, as shown in Fig. 1A. These results show that the activity of Scy-tRNA synthesis is present in cytosol in the soluble state. The fractions of microsomes, mitochondria and nuclei did not contain any activity of Scy-tRNA synthesis.

The spot of Scy on the TLC in Fig. 1 was not clear. By-products were smeared over all areas of the TLC plates. Therefore, we purified Hse^- from the 'H_2Se mixture' by chromatography on Sephacryl S-200, as shown in Fig. 2. Two peaks were observed in the figure and were analyzed by TLC, as shown on the right in Fig. 2. The peaks in fractions 35–38 and 41–46 were glutathione selenotrisulfide and HSe^-, respectively [13]. These two substances were studied as Se donors in the synthesis of Scy-tRNA, as shown in Fig. 3. Fig. 3A showed that a clear Scy spot was found when HSe^- and suppressor tRNA were used. Thus, HSe^- was a good substrate. Glutathione selenotrisulfide was not a Se donor in the synthesis of Scy-tRNA, because there is no Scy spot denoted by the arrow in Fig. 3B. Fig. 3C denotes the case of major serine tRNA instead of suppressor tRNA; a [^{75}Se]Scy spot is not found at the position of the authentic Scy. These results show that HSe^- and the suppressor tRNA were substrates in the synthesis of Scy- tRNA, but major serine tRNA and glutathione selenotrisulfide could not function as substrates. Scy-tRNA was precipitated by the addition of

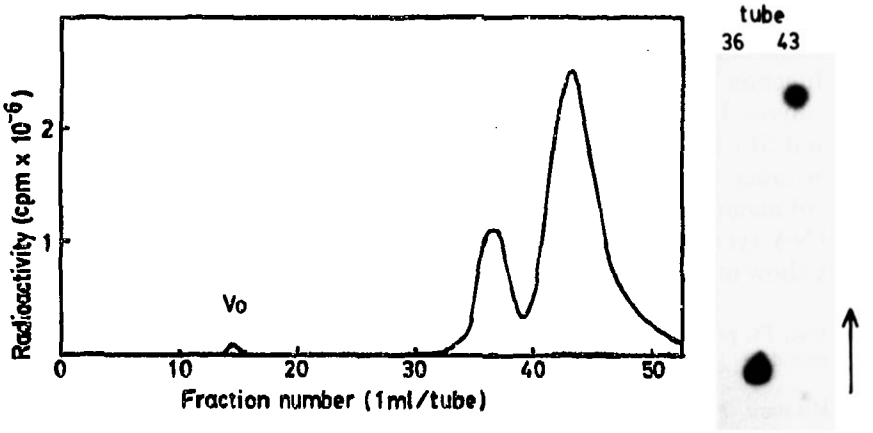

Fig. 2. Chromatographic pattern of 'H_2Se mixture' on Sephacryl S-200. The fractions in tubes 35–38 and tubes 41–46 were analyzed by TLC, and autoradiograph of the TLC plate is shown at the right.

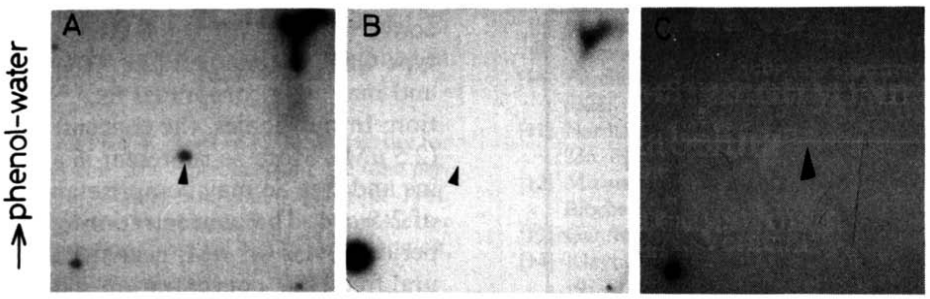

Fig. 3. Autoradiograph of the hydrolyzate of [^{75}Se]aminoacyl-tRNA on silica gel G plates. Arrows indicate the position of cold Scy. The enzyme used was the supernatant at 105 000 × g. A, the product with suppressor tRNA and HSe^-; B, the product with suppressor tRNA and glutathione selenotrisulfide; C, the product with major serine tRNA and HSe^-.

ethanol, and Scy was found in the precipitate of tRNA. We also analyzed the supernatant of ethanol precipitation, but the supernatant did not contain any Scy. This revealed that free Scy was neither synthesized in the reaction mixture nor directly transferred to the suppressor tRNA by some aminoacyl-tRNA synthetase.

Fig. 1 shows that the Scy synthase is present in cytosol. The supernatant at 105 000 × *g* also contains all soluble components, such as tRNA and enzymes. Therefore, we attempted the separation of the Scy synthase from the tRNA by chromatography. The upper portion of Fig. 4 shows an elution pattern of the supernatant on DEAE-cellulose. SerRS activity was found at tube 33. The activity of the Scy synthase was searched for in those eluated fractions and was found in fractions 30–32, as shown in the lower section of Fig. 4. The lower section of Fig. 4 indicates the results of autoradiography of [^{75}Se]Scy on a TLC plate. The relative values of the densitometric tracing of autoradiography were plotted in the upper section of Fig. 4. The Scy synthase was eluted at a slightly lower concentration of KCl (0.17 M) than SerRS (0.20 M). The peak of tRNA kinase was eluted at a higher concentration of KCl than was the SerRS. This showed that the Scy synthase differed from SerRS and tRNA kinase. This fraction (tubes 30–32 in Fig. 4) contained a trace amount of SerRS but did not contain any tRNA kinase activity (data not shown). The fraction without tRNA kinase exhibited activity with which to synthesize Scy-tRNA. This is a key point of this report, because this result shows that Ps-tRNA was not an intermediate in the synthesis of Scy-tRNA. It had been incorrectly considered that Ps-tRNA was an active intermediate in the synthesis of Scy-tRNA; however, this misunderstanding must now be abandoned. This Scy synthase activity was stable during long-time storage in 50% glycerol, 10 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM mercaptoethanol at −80°C.

Scy was found in the tRNA precipitate with ethanol, but was not found in the supernatant. Therefore, Scy is bound to tRNA, as was confirmed by gel chromatography on Sephacryl S-200. The precipitate by ethanol was applied to the column. The result is shown in Fig. 5. A peak of ^{75}Se (tubes 15–19) was found coincidentally with the position of the tRNA. The radioactivity in the peak was confirmed to be from Scy by TLC (data not shown). Thus, we showed that mammalian cells contained the activity of Scy-tRNA synthesis in cytosol and that the Se donor in the synthesis of Scy-tRNA in HSe^-. This HSe^- may be activated by ATP and then transferred to the Ser–tRNA–enzyme complex to become Scy- tRNA, as in the *E. coli* system.

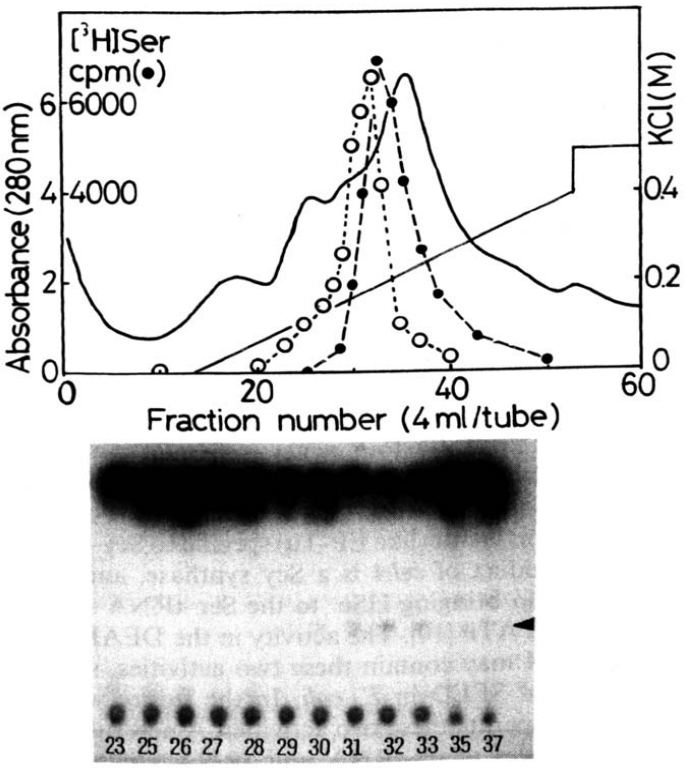

Fig. 4. Chromatograph of the supernatant of murine liver extracts at 105 000 × *g* on DEAE-cellulose. The upper section is a chromatographic profile on DEAE-cellulose. Open circles indicate the density of Scy on the lower autoradiograph. Closed circles indicate SerRS activity (cpm/5 min·5 μl). The lower section is an autoradiograph of Scy liberated from Scy-tRNA produced with the upper eluates as enzyme sources. Arrows indicate the position of Scy.

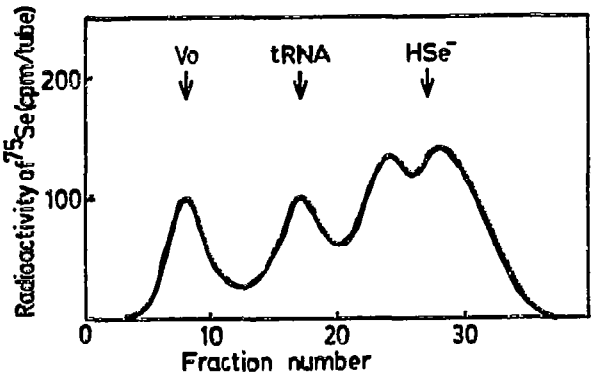

Fig. 5. Chromatographic pattern of the aminoacyl-tRNA precipitate on Sephacryl S-200. The ethanol precipitate of [^{75}Se]Scy-tRNA from the reaction mixture was applied to a column of Sephacryl S-200 in 0.15 M NaCl, 10 mM acetate at pH 4.6.

4. DISCUSSION

Suppressor seryl-tRNA was phosphorylated by tRNA kinase to Ps-tRNA [15]. Suppressor seryl-tRNA is a substrate of this phosphorylation. We purified tRNA kinase from bovine liver [7], and the amount of Ps-tRNA was 5% of the total seryl-tRNA by in vitro phosphorylation [9]. It is therefore considered that this Ps-tRNA must play an important role in cells. This Ps-tRNA was not a substrate for phosphoserine aminotransferase [16]. Since suppressor seryl-tRNA was a precursor of Scy-tRNA, it was considered that Ps-tRNA was an intermediate substance in the process from seryl-tRNA to Scy-tRNA [5,8]. In the previous report [9], we misunderstood Ps-tRNA to be an intermediate, because Scy-tRNA was synthesized in the presence of ATP from seryl-tRNA. However, in this report, we have shown that Scy-tRNA was synthesized from seryl-tRNA in the absence of tRNA kinase; therefore, Ps-tRNA is not an intermediate between seryl-tRNA and Scy-tRNA. ATP should be necessary at an unknown step in order to synthesize Scy-tRNA. Thus, it is concluded that Scy-tRNA synthesis proceeds according to the same mechanism as that in *E. coli*.

In *E. coli* there are four genes (*selA, B, C* and *D*) relating to the synthesis of FDH. The product of *selC* is a suppressor tRNA for Scy [17]. The product of *selB* is a translation factor (like EF-Tu) specific to Scy-tRNA [18]. The product of *selA* is a Scy synthase, and *selD* plays a role in bringing HSe^- to the Ser–tRNA–SELA complex with ATP [10]. The activity in the DEAE fraction in Fig. 4 may contain these two activities, similar to SELA and SELD in *E. coli*. In the future we will attempt to separate these two activities. A new question about the roles of Ps-tRNA and tRNA kinase thus arises. We expect that, in the future, the UGA codon corresponding to phosphoserine will be found in some mRNAs of important proteins.

Se is an essential trace element in humans [19]. We ingest Se as SeO_2 from food and water (the organic compound is converted to SeO_2 by digestion). SeO_2 is converted to HSe^- in the cytosol, where the reductive-type GSH is abundant [20]. HSe^- is used as a Se donor and may be incorporated into Scy-tRNA after activation. In our bodies, the concentration of Se is 0.2 ppm (2.5 μM). More Se is present in a bound state on proteins and free Se may comprise approximately one-tenth of 2.5 μM. The concentration of HSe^- used in this experiment was 0.1 μM, near the above value of the natural free-HSe^- concentration. Therefore, the conditions in this experiment are natural and similar to those in cell cytosol.

The mechanisms of Scy-tRNA synthesis in mammals are the same as those in *E. coli*. In eukaryotes and prokaryotes, there is a natural opal suppressor tRNA specific for Scy. This tRNA is aminoacylated with serine, and then Ser-tRNA is converted to Scy-tRNA by some enzymes in common mechanisms. There may be a translation factor (antireleasing factor) specific for Scy-tRNA in mammals, similar to SELB in *E. coli* [18]. This factor transports Scy-tRNA to ribosomes, and Scy on Scy-tRNA is co-translationally transferred to peptides on ribosomes. Thus, eukaryotes and prokaryotes must have a common Scy translating mechanism. However, the structure of Scy tRNA differs between mammals and *E. coli*, as do the differences between other tRNAs specific for each amino acid. Proteins also differ amongst themselves (GSHPx and type-I iodothyronine deiodinase in eukaryotes; FDH in *E. coli*; glycine reductase in *Clostridium sticklandii*) [8]. Another interesting difference between eukaryotes and prokaryotes involves the secondary structure of mRNA (context effect). The region downstream of the UGA in FDH mRNA forms a specific secondary structure in order to incorporate Scy into FDH on the translational level [21]. However, no common secondary structure downstream of the UGA (Scy codon) among some GSHPx mRNAs and type-I iodothyronine deiodinase mRNA can be pinpointed by a computer search. Mechanisms of discrimination between the Scy codon UGA and the opal termination codon UGA are not clear; thus, this is a very interesting mystery yet to be resolved.

Aminoacyl-tRNA synthetase arose early in evolution and is the oldest of all proteins [22]. However, GSHPx is also an old enzyme, judging from its simple amino acid composition [23]. Thus, the system of Scy incorporation might have had an extensive role in the early stages of the reductive atmosphere of the earth. The UGA codon might not have been a termination codon, but rather a Scy codon in that early stage of the earth. The role of the UGA codon may have changed to that of a termination codon after the stage of the oxidative atmosphere of the earth. According to evolutionary theories, the use of UGA as a termination codon was increased because UGA comprises 50% of the total termination codons in mammals and 27% in *E. coli*. Thus, the pairing of Scy-tRNA and certain seleno-

proteins is essential to sustain life in mammals and *E. coli* at present, but may be come a vestigial remnant in the translation system.

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REFERENCES

[1] Forstrom, J.W., Zakowski, J.J. and Tappel, A.L. (1978) Biochemistry 17, 2639.
[2] Berry, M.J., Banu, L. and Larsen, P.R. (1991) Nature 349, 438–440.
[3] Chambers, I., Frampton, J., Goldfarb, P., Affara, N., McBain, W. and Harrison, P.R. (1986) EMBO J. 5, 1221–1224.
[4] Hatfield, D. and Portugal, F.H. (1970) Proc. Natl. Acad. Sci. USA 67, 1200–1206.
[5] Lee, B.J., Worland, P.J., Davis, J.N., Stadtman, T.C. and Hatfield, D. (1989) J. Biol. Chem. 264, 9724–9727.
[6] Sunde, R.A. and Evenson, J.K. (1987) J. Biol. Chem. 262, 933–937.
[7] Mizutani, T. and Hashimoto, A. (1984) FEBS Lett. 169, 319–322.
[8] Stadtman, T.C. (1990) Ann. Rev. Biochem. 59, 111–127.
[9] Mizutani, T. (1989) FEBS Lett. 250, 142–146.
[10] Forchhammer, K. and Böck, A. (1991) J. Biol. Chem. 266, 6324–6328.
[11] Narihara, T., Fujita, Y. and Mizutani, T. (1982) J. Chromatogr. 236, 513–518.
[12] Mizutani, T., Narihara, T. and Hashimoto, A. (1984) Eur. J. Biochem. 143, 9–13.
[13] Ganther, H.E. (1971) Biochemistry 10, 4089–4098.
[14] Klayman, D.L. and Griffin, T.S. (1973) J. Am. Chem. Soc. 95, 197–200.
[15] Mäenpää, P.H. and Bernfield, M.R. (1970) Proc. Natl. Acad. Sci. USA 67, 688–695.
[16] Tachibana, Y. and Mizutani, T. (1988) Chem. Pharm. Bull. 36, 4019–4025.
[17] Leinfelder, W., Zehelein, E., Mandrand-Berthelot, M. and Böck, A. (1988) Nature 331, 723–725.
[18] Forchhammer, K., Leinfelder, W. and Böck, A. (1989) Nature 342, 453–456.
[19] Keshan Disease Research Group (1979) Chin. Med. J. 92, 477–482.
[20] Murphy, M.E. and Kehrer, J.P. (1989) Biochem. J. 260, 359–364.
[21] Zinoni, F., Heider, J. and Bock, A. (1990) Proc. Natl. Acad. Sci. USA 87, 4660–4664.
[22] Schimmel, P. (1991) Trends Biochem. Sci. 16, 1–2.
[23] Goldfarb, P.S. (1988) Nature 336, 429.