Possible incorporation of phosphoserine into globin readthrough protein via bovine opal suppressor phosphoseryl-tRNA

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Suppressor [^{32}P]phosphoseryl-tRNA, prepared using bovine seryl-tRNA synthetase and ATP:seryl-tRNA phosphotransferase, was mixed with rabbit reticulocyte lysates containing endogenous hemoglobin mRNA having the termination codon UGA (opal). The chromatographic pattern of the lysate on Sephacryl S-200 showed that the radioactivity of [^{32}P]phosphate in the hot trichloroacetic acid-precipitate (phosphoprotein) was eluted at the position between mature hemoglobin and globin subunits. The phosphoprotein, obtained by chromatography on S-200, moved to the position corresponding to that of globin readthrough protein on SDS-PAGE. The analyses of the hydrolyzate of the phosphoprotein showed the presence of phosphoserine in the protein. These results suggest that animal opal suppressor tRNA functions in vitro to transfer phosphoserine to the position of the termination codon UGA (opal) on mRNA.

Phosphoserine *Phosphoprotein* *Opal suppressor* *Seryl-tRNA* *Readthrough protein* *(Rabbit reticulocyte lysate)*

1. INTRODUCTION

It has been reported that one $tRNA^{Ser}$ in higher vertebrate, having CmCA as the anticodon, corresponds to the opal termination codon (UGA) as suppressor tRNA [1], and that this tRNA has a suppressor function, confirmed by the production of hemoglobin readthrough protein [2]. This seryl-tRNA was phosphorylated with ATP:seryl-tRNA phosphotransferase (tRNA kinase) to yield phosphoseryl-tRNA (Ps-tRNA) [3,4]. During the course of studying the role of Ps-tRNA, we purified tRNA kinase [5] and seryl-tRNA synthetase (SerRS) [6] from bovine liver. The tRNA kinase did not catalyze major seryl-$tRNA^{Ser}_{IGA}$ but suppressor seryl-$tRNA^{Ser}_{CmCA}$ [5], so the recognition sites on tRNA by tRNA kinase must be present at different regions (the aminoacyl and anticodon stems) between these two tRNAs [7]. These regions are coincident with the different regions between *E. coli* $tRNA_f^{Met}$ and $tRNA_m^{Met}$, which are recognized by transformylase. Meanwhile, SerRS recognized three $tRNAs^{Ser}$, namely $tRNA^{Ser}_{GCU}$, $tRNA^{Ser}_{IGA}$ and $tRNA^{Ser}_{CmCA}$ with the same affinity and velocity [6]. Recently, it has been reported that the human opal suppressor tRNA gene has a TCA anticodon [8] as does the chicken suppressor tRNA gene [9]. Meanwhile, wheat germ $tRNA^{Trp}$ has CmCA as the anticodon sequence [10].

Many biochemists have become interested in the phosphorylation of proteins in relation to biological functions such as second messenger and tumor promotion [11,12]. It has been suggested that apo-B in VLDL is synthesized as Ps-containing protein [13]. Therefore, it may be interesting to use Ps-tRNA in the synthesis of phosphoprotein. It has been reported that β-globin readthrough protein, which is 23 amino acids longer than native globin and is terminated at the UAA codon, is produced with endogenous suppressor $tRNA^{Trp}$ [14] or with yeast mitochondrial $tRNA^{Trp}$ [15]. However, the direct incorporation

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of tryptophan from Trp-tRNA into the newly synthesized proteins was not shown by these experiments. The incorporation of serine into globin readthrough protein via suppressor Ser-tRNA was not confirmed, but the radioactivity of [^{35}S]methionine on SDS-PAGE was found at the corresponding position of globin readthrough protein [2]. Here, we show the incorporation of labeled Ps into readthrough proteins via bovine suppressor Ps-tRNA in rabbit reticulocyte lysates [16] using endogenous β-globin mRNA [17], which contains the opal termination codon UGA.

2. EXPERIMENTAL

Bovine SerRS, tRNA kinase and bovine suppressor tRNA^{Ser} were prepared according to [5,6,18]. [^{32}P]ATP was synthesized from carrier-free [^{32}P]phosphate as in [19]. [^{14}C]Serine and [^{14}C]leucine were products of the Radiochemical Center (Amersham).

The preparation of [^{32}P]Ps-tRNA was as follows: Suppressor Ser-tRNA was prepared by the aminoacylation of tRNA$^{Ser}_{CmCA}$ (0.5 mg, specific content 1.2 nmol/mg) with SerRS in the presence of 5 mM ATP [6], and then the pH value of the reaction mixture was adjusted to pH 4.6 with acetate buffer. Ser-tRNA was precipitated by the addition of 2 vols ethanol in order to remove cold ATP. The precipitate was washed with ethanol and dried with ethanol and ether. Ser-tRNA was dissolved in 0.5 ml of 10 mM Hepes buffer at pH 6.4 and 10 mM $MgCl_2$ and phosphorylated with 0.1 mg tRNA kinase in the presence of 0.5 mCi [^{32}P]ATP (approx. 500 Ci/mmol) for 30 min at 30°C. Then the pH value of the mixture was adjusted to pH 4.6 with acetate buffer and Ps-tRNA was precipitated by the addition of 2 vols ethanol. The precipitate containing [^{32}P]Ps-tRNA was dissolved in 0.1 ml of 10 mM acetate buffer at pH 4.6 and the solution was chromatographed on a column (50 × 1 cm) of Sephacryl S-200. [^{32}P]Ps-tRNA, eluted at K_{av} = 0.3, was collected and precipitated with ethanol and dried. [^{32}P]Ps-tRNA containing a radioactivity of about 5 × 10^5 cpm was obtained by this method.

Rabbit reticulocyte lysates were prepared by the administration of phenylhydrazine according to [16]. The lysate solution (100 μl) containing standard reagents [16] was mixed with the dry [^{32}P]Ps-tRNA (5 × 10^5 cpm, 0.2 μCi) and [^{14}C]leucine (0.125 μCi). The incubation was carried out for 30 min at 30°C and then the lysate was applied on a column of Sephacryl S-200 (50 × 1 cm) in 0.2 M NaCl-10 mM Tris-HCl at pH 7.6 and 5 mM nitrilotriacetic acid as an inhibitor of phosphatase. For the measurement of radioactivity in the eluate from the column, a part of the eluate was spotted on a filter paper and counted. Sometimes the filter papers were treated with cold 5% trichloroacetic acid or hot 5% trichloroacetic acid in order to estimate the amounts of phosphate on proteins or Ps-tRNA. SDS disc gel electrophoresis was carried out on 15% gel for determination of the molecular mass of the proteins [20]. The gel was stained with Coomassie brilliant blue to detect the globin position and then the gel was sliced at 5 mm sections and incubated with 0.5 ml Protosol (New England Nuclear). The gel slices were counted in a standard toluene/PPO/POPOP solution with a Searle analytical liquid scintillation counter.

The phosphoamino acid in the phosphoproteins was analyzed as follows: The proteins were precipitated with 5% trichloroacetic acid and the precipitate was collected and washed with ethanol. The dry proteins were hydrolyzed in 6 N HCl at 100°C for 3 h. After HCl was removed by evaporation, the hydrolysate was used for analyses. Chromatography on a column (10 × 0.3 cm) of AG-1 was done by elution of a gradient made of 2 mM HCl (35 ml) and 50 mM HCl (20 ml) [21]. Paper electrophoresis was carried out at pH 2.0 (formic acid/acetic acid/water, 1:2:45) at 600 V and 20 mM for 2 h [22].

3. RESULTS AND DISCUSSION

The incorporation of [^{32}P]phosphate into proteins in the presence of uncharged tRNA^{Ser}, serine, [^{32}P]ATP, SerRS and tRNA kinase in rabbit reticulocyte lysates was not effective. Nevertheless, it is possible that some proteins are phosphorylated through [^{32}P]ATP by protein kinases in the lysate system. Therefore, we carried out the experiments using [^{32}P]Ps-tRNA which was prepared as described in section 2. The incorporation of [^{32}P]Ps from [^{32}P]Ps-tRNA in the precipitate of rabbit reticulocyte lysates with hot 5% trichloroacetic acid was increased according to the incubation time. Analyses of the lysates incubated

in the presence of [^{32}P]Ps-tRNA showed that the main band of ^{32}P was found at a larger molecular mass (18 kDa) than that of the globin subunits (16 kDa). The molecular mass of 18 kDa is consistent with that of the globin readthrough protein. This result suggests that [^{32}P]phosphate through [^{32}P]Ps-tRNA was incorporated into the readthrough protein.

To clarify the incorporation, the lysate, which was incubated in the presence of [^{32}P]Ps-tRNA, was fractionated by gel-exclusion chromatography on Sephacryl S-200 as shown in fig.1, which contains the patterns of radioactivity of ^{32}P and ^{14}C in the precipitate of eluate with hot 5% trichloroacetic acid. Unincorporated free [^{14}C]leucine and free ^{32}P were eluted at the V_i position in fig.1, even though these patterns are not shown. Fig.1 also contains the pattern of protein concentration determined by the standard Lowry method. The pattern is similar to that of the absorbance measured at 520 nm (hemoglobin). The peak of [^{14}C]leucine in fig.1 was identical with the position of the endogenous cold hemoglobin of molecular mass 64 kDa. The pattern of [^{14}C]leucine shows another broad peak at tubes 63–68 and this peak should contain immature hemoglobin subunits (16 kDa).

In fig.1, a major peak of ^{32}P was found in tubes 57–61 (fraction B). This incorporation of [^{32}P]Ps was found in proteins because the radioactivity remained in the precipitate after the treatment with hot 5% trichloroacetic acid. Thus, the readthrough [^{32}P]phosphoprotein was found as one peak of a globin subunit and this [^{32}P]Ps-globin subunit was eluted at a slightly larger position than [^{14}C]globin subunits. The results also showed that the globin readthrough protein containing [^{32}P]Ps was not incorporated into the mature hemoglobin of peak A in fig.1, because peak A did not contain ^{32}P radioactivity. This result suggests that the readthrough protein did not fit in the highly ordered structure of mature hemoglobin.

[^{32}P]Ps-proteins in peak B in fig.1 were analyzed by SDS disc gel electrophoresis and the result is shown in fig.2. The peak of [^{32}P] in fig.2 (indicated by an open arrow) was found at the position of the globin readthrough protein (18 kDa), because the readthrough protein should be 23 amino acids larger than globin [23]. The peak of [^{14}C]leucine was coincident with the position of native globin subunits of 16 kDa on SDS-PAGE.

The presence of [^{32}P]Ps in the hydrolysate of fraction B in fig.1 was analyzed by chromatography on AG-1 and by paper elec-

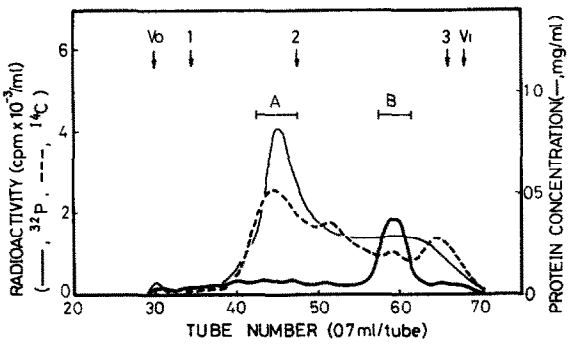

Fig.1. Chromatographic pattern on Sephacryl S-200 of rabbit reticulocyte lysates incubated in the presence of [^{32}P]Ps-tRNA and [^{14}C]leucine. Radioactivity indicated is that in the precipitate of eluate by treatment with hot 5% trichloroacetic acid. The numbers in the figure are the elution points of standard proteins: 1, SerRS (179 kDa); 2, egg albumin (46 kDa); 3, cytochrome *c* (12.5 kDa). V_0 and V_i are void volume of the column and inner volume, respectively.

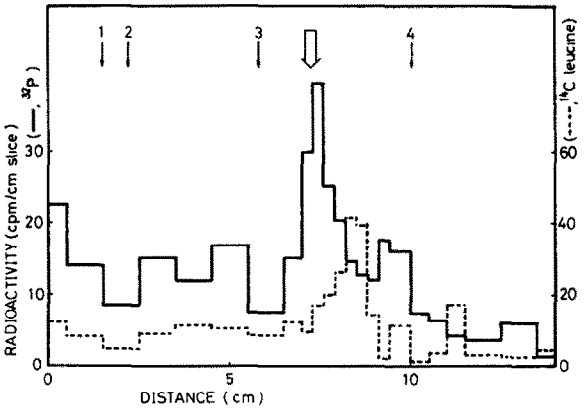

Fig.2. Gel electrophoretic pattern of fraction B in fig.1. The numbers in the figure are the positions of standard proteins: 1, bovine serum albumin (69 kDa); 2, H chain of IgG (50 kDa); 3, L chain of IgG (23 kDa); 4, cytochrome *c* (12.5 kDa). The open arrow indicates the band of globin readthrough protein containing [^{32}P]Ps. The major band of [^{14}C]leucine is normal globin and coincided with the band of standard globin.

trophoresis at pH 2 as shown in fig.3. The results showed that the hydrolysate contained [^{32}P]Ps and [^{32}P]phosphate, which must be liberated from Ps during the hydrolysis with 6 N HCl. These results support the proposal that Ps was incorporated into globin readthrough protein through Ps-tRNA. The ratio of [^{32}P]phosphate incorporated into proteins to total [^{32}P]Ps-tRNA added in the lysates was of a similar level to that of [^{14}C]leucine which was used as a standard amino acid to be incorporated into the newly synthesized proteins. This fact is understood from the value on the ordinate in figs 1 and 2.

It was confirmed that free [^{32}P]Ps was liberated from Ps-tRNA during the incubation of lysates and presented in the supernatant of the lysates treated with cold 5% trichloroacetic acid. It is possible that this free Ps is changed to phosphohydroxypyruvate by phosphoserine transaminase [24]. It is known that the concentration of free Ps is about 5 μM in serum and 100 μM

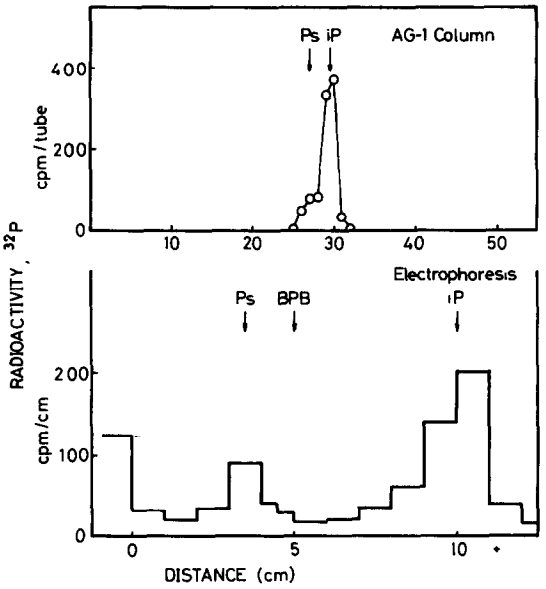

Fig.3. Analyses of phosphoserine in the hydrolysate of readthrough protein (fraction B in fig.1). The upper column shows the results of analyses by chromatography on an AG-1 column and the lower column is that on paper electrophoresis. Ps and iP indicate the eluting positions of authentic Ps and inorganic phosphate, respectively. BPB in the lower column indicates the position of the BPB of a color marker.

in liver. Free Ps might be used as an intermediate of other substances, such as the phosphatidylserine of phospholipids.

The β-hemoglobin readthrough protein was synthesized in the presence of suppressor tRNA^{Ser} [2] and Ps-tRNA well bound to ribosomes [25]. From these two previous results, the incorporation of Ps into the readthrough protein is not strange but conceivable. This report provides evidence to show the incorporation of phosphate into phosphoproteins via phosphoseryl-tRNA. However, there may be unknown mechanisms in the reaction to incorporate phosphate, for example some serine residues on proteins may be phosphorylated using phosphate on phosphoseryl-tRNA, even if the process is not economical. The possibility of unknown mechanisms occurring was not completely excluded in this experiment. Therefore, in future, it will be necessary to confirm the incorporation of phosphoserine at the reasonable position on the β-globin readthrough protein by the analysis of proteolytic digests of phosphoprotein.

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REFERENCES

[1] Hatfield, D. (1985) Trends Biochem. Sci. 10, 201–204.
[2] Diamond, A., Dudock, B. and Hatfield, D. (1981) Cell 25, 497–506.
[3] Maenpaa, P.H. and Bernfield, M.R. (1970) Proc. Natl. Acad. Sci. USA 67, 688–695.
[4] Sharp, S.J. and Stewart, T.S. (1977) Nucleic Acids Res. 4, 2123–2136.
[5] Mizutani, T. and Hashimoto, A. (1984) FEBS Lett. 169, 319–322.
[6] Mizutani, T., Narihara, T. and Hashimoto, A. (1984) Eur. J. Biochem. 143, 9–13.
[7] Gauss, D.H. and Sprinzl, M. (1983) Nucleic Acids Res. 11, r1–r54.
[8] O'Neill, V.A., Eden, F.C., Pratt, K. and Hatfield, D.L. (1985) J. Biol. Chem. 260, 2501–2508.
[9] Hatfield, D.L., Dudock, B.S. and Eden, F.C. (1983) Proc. Natl. Acad. Sci. USA 80, 4940–4944.
[10] Ghosh, K. and Ghosh, H.P. (1984) Nucleic Acids Res. 12, 4997–5003.
[11] Witte, N.O., Dasgupta, A. and Baltimore, D. (1980) Nature 283, 826–831.

[12] Imaoka, T., Lynham, J.A. and Haslam, R.J. (1983) J. Biol. Chem. 258, 11404–11414.
[13] Davis, R.A., Clinton, G.M., Borchardt, R.A., Malone-McNeal, M., Tan, T. and Lattier, G.R. (1984) J. Biol. Chem. 259, 3383–3386.
[14] Geller, A.J. and Rich, A. (1980) Nature 283, 41–46.
[15] Marin, R.P., Sibler, A.P., Dirheimer, G., DeHenau, S. and Grosjean, H. (1981) Nature 293, 235–237.
[16] Suzuki, H. and Hayashi, Y. (1975) FEBS Lett. 52, 258–261.
[17] Efstratiadis, A., Kaftos, F.C. and Maniatis, T. (1977) Cell 10, 571–585.
[18] Narihara, T., Fujita, Y. and Mizutani, T. (1982) J. Chromatogr. 236, 513–518.
[19] Walseth, T.F. and Hohnson, R.A. (1979) Biochim. Biophys. Acta 526, 11–31.
[20] Laemmli, U.K. (1970) Nature 227, 680–682.
[21] Takemura, S., Mizutani, T. and Miyazaki, M. (1968) J. Biochem. 64, 827–837.
[22] Takahashi, S.Y. (1983) Int. J. Invertebr. Reprod. 6, 65–76.
[23] Van Ooyen, A., Van de Berg, J., Mantei, N. and Weissmann, C. (1979) Science 206, 337–344.
[24] Hirsh, H. and Greenberg, D.H. (1967) J. Biol. Chem. 242, 2283–2287.
[25] Hatfield, D., Diamond, A. and Dudock, B. (1982) Proc. Natl. Acad. Sci. USA 79, 6215–6219.