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Note

Characterization of Bovine Heart Sulfotransferase Catalyzing the Sulfation of Tyrosine-Containing Peptides

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Summary Using [^{35}S]PAPS as the sulfate donor, we have detected a sulfotransferase from bovine heart which catalyzes the sulfation of tyrosine-containing peptides. The enzyme displayed optimal activity at pH 5.75 and 35°C in a one-hour reaction. The addition of 10 mM Mn^{2+} or Co^{2+} to the reaction mixture increased the sulfotransferase activity by 3.4- and 3.5-fold, respectively. In contrast, the maximum increment stimulated by Mg^{2+} was only 1.75-fold at 15 mM concentration, and instead of exerting an enhancement effect, Ca^{2+} was found to be a potent inhibitor. The addition of 50 mM NaF to the reaction mixture resulted in an increase in sulfotransferase activity of 3.3-fold. The Km for 3′-phosphoadenosine 5′-phosphosulfate (PAPS) was determined to be 2 μM at a constant 0.5 mM Boc-Glu-Asp-Tyr-Val. Among the 10 peptides tested as substrates, Boc-Glu-Asp-Tyr-Val and Boc-Asp-Asp-Tyr-Val provided the highest activities.

Key Words sulfotransferase, bovine heart, tyrosine sulfation

In the metabolic assimilation of sulfur from inorganic sulfate present in foods or as a degradation product of methionine or cysteine, the activation of sulfate is an obligate step following its entry into cells. Sulfate activation takes place in two consecutive reactions catalyzed by adenosine 5′-triphosphate (ATP) sulfurylase (EC 2.7.7.4) and adenosine 5′-phosphosulfate (APS) kinase (EC 2.7.1.25). The "active sulfate," 3′-phosphoadenosine 5′-phosphosulfate (PAPS) is the sulfate donor for all sulfation reactions that occur in mammalian cells. Therefore, this nucleotide is indispensable for the study of sulfotransferase enzymes.

Recent studies have revealed the widespread occurrence of post-translational

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Abbreviations: PAPS, 3′-phosphoadenosine 5′-phosphosulfate; Boc, butyloxy carbonyl; PMSF, phenylmethylsulphonyl fluoride; 5′-AMP, 5′-adenosine monophosphate.

protein tyrosine sulfation among secretory proteins, as well as integral membrane proteins, produced by multicellular eukaryotic cells (*1*). Consistent with the pathway of the biosynthetic transport of these proteins, the enzyme, designated tyrosylprotein sulfotransferase (TPST), that catalyzes the sulfation reaction has been shown to be located in the Golgi (*2–4*). Although studies have been performed to characterize TPST from a wide range of cells and tissues (*5–12*), no reports are currently available on TPST from muscle tissues. In view of the fact that the heart produces some hormones whose biosynthetic precursors may potentially be subjected to post-translational tyrosine sulfation, we decided to investigate the presence of TPST in bovine heart and characterize some of its properties.

Materials and methods

[^{35}S]PAPS was prepared based on a procedure previously established (*13*). Peptides used as substrates in the enzymatic reactions were synthesized according to the method developed by Bodanszky and Bodanszky (*14*). All other chemicals used were of the highest grades commercially available.

The bovine heart Golgi-enriched microsomal membranes were prepared by methods modified from those developed by Walter and Blobel (*15*) and Trifaró and Duerr (*16*). Briefly, bovine heart muscle was homogenized in 5 volumes of buffer A (100 mM sodium phosphate (pH 6.25), 5 mM $MgCl_2$, 100 mM NaCl, 5 mM 2-mercaptoethanol, 1 mM PMSF, 0.1 mM iodoacetic acid and 2 kIU/mL aprotinin) containing 0.5 M sucrose. The microsomal membranes were collected on a cushion of 1.3 M sucrose upon density gradient centrifugation at 100,000×*g* for 60 min. Subsequently, the Golgi-enriched membranes were isolated from a 0.5/1.1 M sucrose interface upon centrifugation at 144,000×*g* for 2.5 h, and washed successively with buffer B (50 mM HEPES (pH 7.0), 1 mM EDTA, 5 mM 2-mercaptoethanol, 1 mM PMSF, 0.1 mM iodoacetic acid and 2 kIU/mL aprotinin) containing 1 M NaCl and buffer B containing 0.5% (w/v) Triton X-100 and 10% (w/v) glycerol. The Golgi-enriched microsomal membranes thus obtained were solubilized in buffer B containing 1% (w/v) Triton X-100 plus 20% (w/v) glycerol, and used as the source of sulfotransferase in the subsequent characterizations. The α-mannosidase II activity, which has been recognized as a Golgi membrane marker, was assayed for monitoring the distribution of Golgi membranes in the various centrifugal fractions using the procedure of Tulsiani et al (*17*).

The standard sulfotransferase assay was performed using 0.5 mM Boc-Glu-Asp-Tyr-Val and 4 μM [^{35}S]PAPS in 100 μL of a reaction mixture containing 50 mM MES (pH 5.75), 50 mM NaF, 10 mM $MnCl_2$, 1 mM 5′-AMP and 0.3% (w/v) Triton X-100. The reaction was started by the addition of the enzyme preparation, allowed to incubate at 35°C for 1 h and terminated by heating at 90°C for 3 min. The [^{35}S]sulfated peptide generated was isolated by reverse phase HPLC on a Chromatorex-ODS column (4.6×150 mm i.d.) with CH_3CN in aqueous 1% (v/v) TFA (adjusted to pH 2.5 with diethylamine) as the mobile phase. The collected [^{35}S]sulfated peptide was analyzed by liquid scintillation counting.

Results and discussion

Protein tyrosine sulfation is a widespread post-translational modification that occurs in many functional and structural proteins (*1*). In this study, we characterized the TPST from the bovine heart Golgi membranes with emphasis on substrate sequence specificity.

To determine the linearity of bovine heart sulfotransferase activity, reactions were carried out with 0.5 mM Boc-Glu-Asp-Tyr-Val, as described for the standard assay, for up to 2 h. The reaction was found to be linear up to 60 min at 35°C. However, there was marked loss of activity when incubated for more than 1 h. Therefore, for all other determinations, the reactions were performed for 1 h. The optimum pH for the bovine heart TPST was determined to be 5.75, which is in accord with the pH optima reported for TPSTs from other tissues (*5–12*). It was noted that MES buffer was preferred to maleate buffer (40–50% of the activity detected in MES buffer). The optimum temperature for a one-hour reaction was 35°C. The thermostability of the bovine heart TPST was assayed by incubating the enzyme preparation at varying temperatures for 30 min prior to measuring the activity. TPST showed a gradual loss of activity at temperatures above 30°C. Furthermore, there was an almost total loss of activity at 45°C. The effects of NaF, a phosphatase inhibitor previously shown to be effective in preventing the degradation of PAPS by endogenous phosphatase (*7*), on bovine heart TPST activity were tested. Results obtained showed that NaF at 25 mM and 50 mM concentrations increased TPST activity by 2.6- and 3.3-fold, respectively, from the basal level. In contrast, the same concentrations of NaCl showed virtually no enhancement effects. The affinity of bovine heart TPST for PAPS was analyzed in the presence of 0.5 mM Boc-Glu-Asp-Tyr-Val. The Km of PAPS was calculated from the Lineweaver Burk plot, and was found to be 2 μM, indicating that bovine heart TPST has a slightly lesser affinity for PAPS than bovine liver TPST (Km = 1.38 μM). The heart TPST was inhibited by a submicromolar (10^{-8} M) level of 2,6-dichloro-4-nitrophenol (DCNP). The distribution of TPST in the bovine heart microsomal membranes was investigated by measuring the TPST activity in the fractionated membranes along with the assay for α-mannosidase II, a Golgi marker enzyme. The highest activity of TPST was in the Golgi-enriched membranes.

Figure 1 shows the effects of divalent cations on bovine heart sulfotransferase activity. The heart TPST was stimulated 3.4- and 3.5-fold in the presence of 10 mM Mn^{2+} or Co^{2+}, respectively. In contrast, Mg^{2+} stimulated TPST activity by only 1.75-fold at a 15 mM concentration. Ca^{2+}, on the other hand, was found to be a potent inhibitor of the enzyme, inhibiting 92.5% of its activity at a 10 mM concentration. It has previously been reported that TPSTs in many tissues can be activated by Mn^{2+} and Mg^{2+} (*5–7*). For the rat liver enzyme, Co^{2+} was only 25% as effective as Mn^{2+} (*7*). In our experiments, Co^{2+} was equally as effective as Mn^{2+} in stimulating bovine heart TPST. It is to be pointed out that, despite the enhancement effects described above, divalent cations appeared not to be absolutely required since basal activity was still detected without divalent cations in the

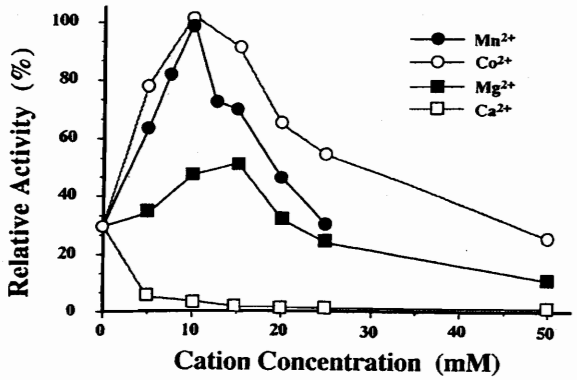

Fig. 1. Effects of divalent cations on bovine heart sulfotransferase activity. Reactions were carried out using 0.5 mM Boc-Glu-Asp-Tyr-Val and 2 μM [^{35}S]-PAPS as co-substrates in 50 mM MES containing 50 mM NaF, 1 mM 5′-AMP and varying concentrations of the divalent cations tested for 1 h at 35°C.

reaction mixture.

A database analysis of the tyrosine sulfation sites in proteins (*18*) has revealed certain consensus features in the neighboring amino acid sequences. These include an abundance of acidic amino acids, at least one of which is on the N-terminal side of the sulfated tyrosine residues (*19*), presence of turn-inducing amino acids in the vicinity, the absence of neighboring cysteine residues and the lack of secondary structures such as N-glycosylation sites near the sulfated tyrosine residue. Since these consensus features are known to govern the tyrosine sulfation sites, it is useful to study the substrate specificity at the sequence level of the bovine heart TPST. For this purpose, TPST activities toward a number of synthetic peptides instead of EAY, a synthetic random polymer, with varying amino acid sequences flanking the tyrosine residue were analyzed. The sulfation of synthetic peptides was confirmed by co-elution of the radiolabeled peptides and the cold sulfated peptides upon HPLC.

Figure 2 shows the relative activities (reaction velocity pmol/h/mg) of bovine heart sulfotransferase with various synthetic peptides as substrates. Results revealed that the two peptides, Boc-Asp-Asp-Tyr-Val and Boc-Glu-Asp-Tyr-Val, with acidic amino acids at both -2 and -1 positions relative to the tyrosine residue yielded the highest sulfotransferase activities. The two peptides with acidic amino acids at positions -1 and $+1$, Boc-Asp-Tyr-Asp-Val and Boc-Asp-Tyr-Glu-Val, provided somewhat lower activities. The other 6 peptides displayed considerably lower activities compared with the activities detected for the four peptides mentioned above. A comparison of the effects of the side chain of aspartic acid and that of glutamic acid on bovine heart sulfotransferase activity showed that aspartic acid residues at positions -1 or $+1$ were more favored to glutamic acid residues; whereas this effect was not seen at position -2. These data confirmed that the sulfotransferase from bovine heart also recognized the acidic amino acid residues in the vicinity of the sulfatable tyrosine residue, a part of consensus features, and is

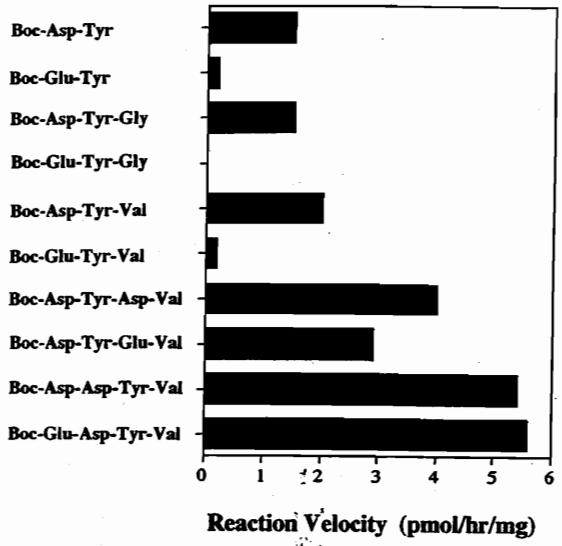

Fig. 2. Substrate specificity of bovine heart sulfotransferase. Reactions were carried out with 0.1 mM peptide substrate and 2 μM [^{35}S]PAPS as co-substrate in 50 mM MES containing 10 mM $MnCl_2$, 50 mM NaF and 1 mM 5′-AMP for 1 h at 35°C.

therefore similar to the TPSTs isolated from rat liver and adrenal gland (*7*).

The heart produces some hormones whose biosynthetic precursors may potentially be subjected to post-translational tyrosine sulfation. To elucidate the nature of the sulfate metabolism, it will be important to study the relation between the biosynthesis of PAPS and the various sulfation reactions that take place *in vivo*.

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