Conversion of Recombinant Hirudin to the Natural Form by in Vitro Tyrosine Sulfation

DIFFERENTIAL SUBSTRATE SPECIFICITIES OF LEECH AND BOVINE TYROSYLPROTEIN SULFOTRANSFERASES*

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Hirudin, a tyrosine-sulfated protein secreted by the leech Hirudo medicinalis, is one of the most potent anticoagulants known. The hirudin cDNA has previously been cloned and has been expressed in yeast, but the resulting recombinant protein was found to be produced in the unsulfated form, which is known to have an at least 10 times lower affinity for thrombin than the naturally occurring tyrosine-sulfated hirudin. Here we describe the in vitro tyrosine sulfation of recombinant hirudin by leech and bovine tyrosylprotein sulfotransferase (TPST). With both enzymes, in vitro sulfation of recombinant hirudin occurred at the physiological site (Tyr-63) and rendered the protein biochemically and biologically indistinguishable from natural hirudin. However, leech TPST had an over 20-fold lower apparent $K_m$ value for recombinant hirudin than bovine TPST. Further differences in the catalytic properties of leech and bovine TPSTs were observed when synthetic peptides were tested as substrates. Moreover, a synthetic peptide corresponding to the 9 carboxyl-terminal residues of hirudin (which include Tyr-63) was sulfated by leech TPST with a similar apparent $K_m$ value as full length hirudin, indicating that structural determinants residing in the immediate vicinity of Tyr-63 are sufficient for sulfation to occur.

Recombinant proteins1 are increasingly being used in biology and medicine. Most of these proteins are secretory, and many of them are post-translationally modified. One post-translational modification found in many secretory proteins is tyrosine sulfation (Huttner and Baeuerle, 1988; Huttner, 1986) which occurs in the lumen of the trans Golgi and is catalyzed by an integral membrane protein, tyrosylprotein sulfotransferase (TPST)2 (Lee and Huttner, 1985; Baeuerle and Huttner, 1987; Niehrs and Huttner, 1990). However, when proteins that are physiologically tyrosine-sulfated are expressed in bacteria and yeast, these recombinant proteins are not tyrosine-sulfated (Riehl-Bellon et al., 1989). This is consistent with the observations that protein tyrosine sulfation, though widespread in metazoan cells, does not occur in prokaryotes and certain lower eukaryotes (Huttner and Baeuerle, 1988; Holmnaun et al., 1985), presumably because of the lack of TPST.3

In several cases, tyrosine sulfation has been shown to be of major physiological importance, affecting the biological activity or half-life of specific proteins (Anastasi et al., 1966; Bodanszky et al., 1978; Nachman et al., 1986; Pauwels et al., 1987; Suiko and Liu, 1988; Hortin et al., 1989). A striking example is the anticoagulant hirudin; the desulfated form of hirudin binds to thrombin with a 10- (Stone and Hofsteenge, 1986) to 15-fold (Seemuller et al., 1986; Dodt et al., 1987) lower affinity than the natural, tyrosine-sulfated form produced by the leech Hirudo medicinalis. In addition, a tyrosine-sulfated carboxyl-terminal desopeptide of hirudin was found to have a 10-fold higher anticoagulant activity than the unsulfated peptide (Maraganore et al., 1989). These differences between tyrosine-sulfated and unsulfated hirudin observed in vitro may well translate into a large increase in antithrombotic efficacy when used in vivo, in analogy to previous results with hirudin containing a single amino acid change (Degryse et al., 1989).

A hirudin cDNA has recently been cloned (Harvey et al., 1986). The recombinant protein has been expressed in yeast and is available in highly purified form (Loison et al., 1986; Riehl-Bellon et al., 1989). Since hirudin, as one of the most potent anticoagulants known, has great potential in medical therapy (Markwardt, 1970; Markwardt et al., 1984, 1988), it would be desirable to convert the recombinant, unsulfated hirudin, which can be obtained much more easily than hirudin isolated from leeches, to the natural, tyrosine-sulfated form. Here we report on this conversion by using homologous (leech) as well as heterologous (bovine) TPST in vitro. Moreover, we show that leech and bovine TPSTs have distinct catalytic properties.

MATERIALS AND METHODS

Recombinant Hirudin and Synthetic Peptides

A recombinant hirudin variant (HV2) was used in which Asn-47 encoded in the cDNA isolated originally (Harvey et al., 1986) has been changed to Lys-47 (Degryse et al., 1989). This recombinant hirudin variant (previously referred to as HV2(Lys-47) (Degryse et al., 1989) and for ease of presentation referred to as rHV2 in this study) has better kinetic properties toward thrombin than HV2(Asn-47) (Degryse et al., 1989). rHV2 was expressed in Saccharomyces

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cerevisiae (Loison et al., 1988) and purified (>95%) as described (Richel-Bellon et al., 1989) prior to use as substrate for in vitro sulfation. Natural, tyrosine-sulfated hirudin was purified from leech glands (peak B1, Footnote 4; see also Richel-Bellon et al., 1989) and corresponds in amino acid sequence to rHV2. The synthetic peptides Hir-(57-65) (KKEIEPEEYLQ) and CCK-(107-115) (KKSAFE-DYEYPSMG) were synthesized as described (Frank and Gausepohl, 1989). Hir-(57-65) corresponds to the carboxy-terminal nine amino acids of hirudin; CCK-(107-115) corresponds to the carboxy-terminal peptide derived from the cholecytokinin precursor (Adrian et al., 1986; Eng et al., 1986). Both peptides contained at their amino terminus 2 additional lysine residues (not present in the parent protein) which were added to facilitate their electrophoretic separation from [35S]PAPS (Niehrs et al., 1989), CCK-(107-115) also contained additional lysine residues (Met, Glu) at its carboxy terminus for technical reasons related to its synthesis.

**TPST Preparations**

All steps were performed at 4 °C.

**Leech TPST Preparation**—Salivary glands of leeches (H. medicalis, obtained from Ricarimpex, Audenge, France, or from a local technical reason related to its synthesis.

**Tyrosine Sulfation of Recombinant Hirudin**

Thrombin-Sepharose chromatography and Tyrosine Sulfate Analysis

Thrombin-Sepharose chromatography of recombinant hirudin was performed as described previously (Harvey et al., 1966). Tyrosine sulfate in recombinant hirudin sulfated in vitro was analyzed after 10 min hydrolysis and cellulose thin-layer electrophoresis at pH 3.5 (Huttner, 1984). Tyrosine [35S]sulfate and [35S]rHV2 were quantitated by liquid scintillation counting of the ninhydrin-stained tyrosine sulfate spot and the ninhydrin-stained rHV2 spot, respectively (unsulfated and sulfated rHV2 comigrated in this electrophoretic system).

**RESULTS AND DISCUSSION**

When purified rHV2 was incubated with the sulfate donor [35S]3'-phosphoadenosine 5'-phosphosulfate (PAPS) and a membrane preparation from leech salivary glands enriched in TPST, the i.e. the enzyme that physiologically sulfates hirudin, a sulfated product was formed which on HPLC eluted slightly before the peak of unsulfated rHV2 (Fig. 1A). No such product was found when a soluble fraction of leech salivary glands was used as potential source of TPST or when rHV2 was omitted from the sulfation reaction (not shown). The product of the in vitro sulfation comigrated with natural, tyrosine-sulfated hirudin purified from leeches (Fig. 1A), showing that sulfation alone is sufficient to convert the recombinant protein to a form indistinguishable from natural hirudin. As an alternative to leech TPST, we have tested a heterologous TPST preparation, TPST from bovine adrenal medulla, which has been previously characterized (Lee and Huttner, 1985; Niehrs and Huttner, 1990; Niehrs et al., 1990) and is available in larger amounts than the leech enzyme. The rationale behind using this latter enzyme was the previous observation that protein substrate recognition by TPST has been sufficiently conserved during evolution to allow stoichiometric sulfation of an insect protein by mammalian TPST (Friederich et al., 1988). Incubation of purified rHV2 with [35S]PAPS and a membrane preparation enriched in bovine TPST resulted in the formation of sulfated rHV2 which, like the product of the reaction using leech TPST, comigrated with natural hirudin on HPLC (Fig. 1B).

To investigate whether the [35S]SO4 in rHV2 was linked to tyrosine, we subjected the in vitro sulfated rHV2 to alkaline hydrolysis, a condition in which tyrosine sulfate is released from proteins (Huttner, 1984). Thin-layer electrophoresis of the hydrolysate showed that, indeed, the radioactivity was recovered as tyrosine sulfate (Fig. 2). Serine sulfate and threonine sulfate were not detected.
Hirudin contains 2 tyrosine residues, one at position 3 and one at position 63, 3 residues from the carboxyl terminus (Bagdy et al., 1976). Only tyrosine 63 is sulfated in the leech in vivo (Bagdy et al., 1976). To investigate whether the in vitro sulfation of rHV2 by the leech and the bovine TPST preparation occurred specifically at tyrosine 63, purified [35S]PAPS rHV2 was digested with carboxypeptidase Y at pH 5.5, a condition known to selectively release the carboxyl-terminal amino acids (Chang, 1983). Thin-layer electrophoresis of the digests showed that free tyrosine [35S]sulfate was released (not shown). To demonstrate that the release of tyrosine sulfate was caused by carboxypeptidase Y itself, a control digestion was performed at pH 7.4, a pH at which carboxypeptidase Y is inactive (Hayashi, 1977). Thin-layer electrophoresis of the digests showed that free tyrosine [35S]sulfate was released from [35S]rHV2 digested with either TPST preparation (Fig. 3). Serine sulfate and threonine sulfate were not detected (data not shown). To demonstrate that the release of tyrosine sulfate was caused by carboxypeptidase Y itself rather than by a contaminating endopeptidase, a control digestion was performed at pH 7.4, a pH at which carboxypeptidase Y is inactive (Hayashi, 1977). No significant quantities of tyrosine [35S]sulfate were released from [35S]rHV2 under these conditions (Fig. 3). Thus, in vitro sulfation of recombinant hirudin by either leech or bovine TPST occurred at tyrosine 63.

It has previously been shown that the presence of a sulfate group on tyrosine 63 increases the affinity of hirudin toward thrombin (Stone and Hofsteenge, 1986; Seemuller et al., 1986; Dodt et al., 1987). Since in vitro sulfation of rHV2 occurred specifically at this site, we did not attempt to confirm these observations using the minute amounts of tyrosine-sulfated recombinant hirudin produced under the present in vitro conditions. It was, however, important to ascertain that the conditions of in vitro incubation did not unspecifically impair the biological activity of hirudin. The biological activity of hirudin can be demonstrated qualitatively by its binding to immobilized thrombin (Walsmann, 1981). To determine whether recombinant hirudin retained this property after in vitro incubation, purified [35S]rHV2 was subjected to affinity chromatography on a thrombin-Sepharose column (Table I). All of the applied radioactive rHV2 bound to the column, and 70% could be specifically eluted with the thrombin inhibitor 4-aminobenzamidine. Thus, the in vitro sulfated recombinant hirudin was biologically active.

The results described so far show that a recombinant secretary protein can be converted to the physiological form by performing the appropriate post-translational modification, tyrosine sulfation, in vitro. Although this modification occurs late in the secretory pathway (trans Golgi), this was not necessarily to be expected since a protein purified from the extracellular medium may not a priori have the same structure as in the trans Golgi. Differences in structure between the trans Golgi and the secreted form of a protein result, for example, from post-translational modifications occurring later in the secretory pathway than tyrosine sulfation, such as proteolytic processing (for review, see Steiner et al., 1984) and oligomerization via disulfide bonds (e.g. von Willebrand factor; for review, see Verweij, 1988). Thus, it could not be excluded that the trans Golgi form of a protein is specifically competent to undergo tyrosine sulfation. However, the present results show that TPST can not only sulfate proteins endogenously present in subcellular Golgi-containing fractions, as shown previously (for review see Huttner and Bacurro, 1988), but also full length proteins purified after secretion, implying that complete passage through the eukar-
Tyrosine Sulfation of Recombinant Hirudin

In vitro sulfated recombinant hirudin binds to thrombin. [35S]rHV2 sulfated by the leech TPST preparation and purified through HPLC (gradient I, Fig. 1) was chromatographed on a thrombin-Sepharose column. The flow-through was collected, the column was washed, and [35S]rHV2 was eluted with 4-aminobenzamidine. The radioactivity recovered in the various fractions is given after subtraction of background.

**Table I**

| Protein substrate | Leech TPST | Bovine TPST |
|-------------------|------------|-------------|
| [35S]rHV2        | K_m (mM)  | 0.27        | 0.41        |
|                   | V_max (pmol/min/mg) | 1.3        | 0.61        |

**Table II**

Dissociate assays with four to six different concentrations of each substrate were performed, and the kinetic parameters of TPST were determined from a linear fit (r ≥ 0.95) of a double-reciprocal plot.

The radioactivity recovered in the various fractions is given after subtraction of background.

**Figure 2.** In vitro sulfation of recombinant hirudin occurs on tyrosine. [35S]rHV2 sulfated by the leech TPST preparation and purified through HPLC (gradient I, Fig. 1) was subjected to tyrosine sulfate analysis. An autoradiogram of the cellulose thin-layer sheet is shown. The dashed line indicates the position of the tyrosine sulfate (Tyr(S)) standard detected by ninhydrin staining.

**Figure 3.** Carboxypeptidase Y releases tyrosine sulfate from in vitro sulfated recombinant hirudin. [35S]rHV2 obtained from in vitro sulfation reactions with either the leech or the bovine TPST preparation was purified by paper electrophoresis, and aliquots containing 2205 cpm (leech TPST) or 3283 cpm (bovine TPST) were digested with carboxypeptidase Y at pH 5.5 or 7.4. Released tyrosine [35S]sulfate was separated from [35S]rHV2 by electrophoresis and is expressed as percent of total (sum of [35S] radioactivity present in the tyrosine [35S]sulfate plus [35S]rHV2 spots).

The nine carboxyl-terminal amino acid residues of hirudin, which include the tyrosine sulfation site, are of particular relevance for the inhibitory action of hirudin on thrombin, probably by interacting with a noncatalytic domain of thrombin that binds to fibrinogen (Fenton et al., 1988; Noe et al., 1989). Deletion of these residues increases the apparent K_m value of hirudin 10,000-fold (Degryse et al., 1989). Conversely, the 10–12 carboxyl-terminal amino acid residues (in relatively high amounts) are alone sufficient for inhibition of thrombin-mediated clotting (Krstenansky et al., 1987; Mao et al., 1988; Maraganore et al., 1989). It was of interest to investigate whether the structural requirements for the enzymatic tyrosine sulfation of hirudin were also contained in this part of the protein. We therefore compared the sulfation of full length recombinant hirudin with that of the synthetic peptide Hir-(57–65), corresponding to the nine carboxyl-terminal amino acids of hirudin. For this comparison, we determined the kinetic parameters of leech as well as bovine TPST for these two substrates (Table II). Sulfation of Hir-(57–65) occurred on tyrosine (data not shown). The apparent K_m values of leech and bovine TPST for Hir-(57–65) were in the same range. In contrast, only leech TPST had an apparent K_m for full length hirudin that was similar to that for Hir-(57–65), whereas bovine TPST had a 23-fold higher apparent K_m for full length hirudin than for Hir-(57–65) (Table II). These data suggest that the structural information required for the recognition of hirudin by TPST is contained within the nine carboxyl-terminal residues of hirudin, and that the tertiary structure of full length hirudin does not promote this recognition. Rather, the tertiary structure of full length hirudin can impose steric hindrance on this recognition, unless the TPST is evolutionarily adapted for this substrate, as appears to be the case for leech, but not bovine, TPST.

The different apparent K_m values of leech and bovine TPST for full length hirudin versus Hir-(57–65) suggested that leech and bovine TPST have differential substrate specificities. Differences in catalytic properties between leech and bovine TPST were also observed with respect to V_max, which was in the same range for full length hirudin and Hir-(57–65) in the case of leech TPST but differed 20-fold in the case of bovine TPST (Table II). Moreover, further differences between leech and bovine TPST became apparent when we assayed both enzymes with a second synthetic peptide, CCK-(107–115), corresponding to the carboxyl-terminal sulfation sites of preprocholecystokinin (Adrian et al., 1986; Eng et al., 1986). Leech TPST exhibited a 24-fold lower apparent K_m value for CCK-(107–115) than for Hir-(57–65), with little change in V_max, whereas bovine TPST showed a 16-fold higher V_max for CCK-(107–115) than for Hir-(57–65), with little change in apparent K_m (Table II). Thus, leech and bovine TPST are distinct in their catalytic properties toward two synthetic peptides, although both peptides conform to the previously suggested consensus features for tyrosine sulfation (Huttner et al., 1986; Eng et al., 1986).
and Baeuerle, 1988; Hortin et al., 1986) in that they contain acidic and turn-inducing amino acid residues in the vicinity of the tyrosine. Hence, different types of TPST have evolved in different species. It is tempting to speculate that multiple types of TPST may exist in the same organism.

What perspectives emerge from the in vitro tyrosine sulfation of recombinant hirudin and the comparison of leech and bovine TPSTs? It seems feasible to produce natural-like hirudin for use in medical therapy by enzymatic sulfation of recombinant hirudin, provided that efficient sulfation systems, e.g. using overexpressed TPST in vivo or preparative amounts of TPST in vitro, are available. The latter require the cloning of TPST, which is within reach since we have very recently purified TPST from bovine adrenal medulla over 100,000-fold to apparent homogeneity (Niehrs and Huttner, 1990).

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