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Selective Mechanism-based Inactivation of Peptidylglycine α-Hydroxylating Monooxygenase in Serum and Heart Atrium vs. Brain

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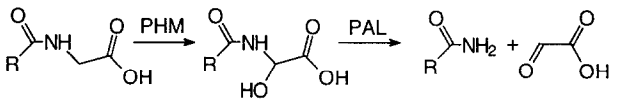
ABSTRACT. Peptidylglycine α-hydroxylating monooxygenase (PHM; EC 1.14.17.3) catalyses the rate-limiting step in the post-translational activation of substance P, among other neuropeptides, from its glycine-extended precursor. Comparative kinetic studies were performed, using *trans*-styrylacetic acid or *trans*-styrylthioacetic acid as known mechanism-based inhibitors, of PHM isolated from rat, horse or human blood serum. Distinctive species differences with respect to PHM inactivation were observed: the efficiency of inactivation decreased in the order of horse ≫ rat > human. *Trans*-styrylacetic acid was more active than its thioether derivative. Moreover, we studied the differential sensitivity towards mechanism-based inactivation, of soluble PHM from rat blood serum and rat brain by *trans*-styrylacetic acid or benzylhydrazine, as well as the membrane-associated enzymes from rat brain and heart atrium. For the heart atrium membrane PHM or the soluble PHM from blood serum, inactivation rate constants k_{inact}/K_I of approximately 100 M^{-1} sec^{-1} were found with *trans*-styrylacetic acid. However, neither of the two tested compounds, at 100 μM or 12 mM, respectively, could inactivate the soluble or membranous PHMs from rat brain during a 15-min pre-incubation period. Instead, under conditions of reversible inhibition, *trans*-styrylacetic acid competitively inhibited the soluble or membrane-associated brain PHM with inhibition constants K_I = 0.6 μM and 1.0 μM, respectively. Organ-selective, time-dependent inactivation of PHM with compounds of the above types might be an important pharmacological tool to control peripheral neuropeptide activation. BIOCHEM PHARMACOL 53;11: 1695–1702, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. peptidylglycine α-amidating monooxygenase (rat, horse, human); *trans*-styrylacetic acid; *trans*-styrylthioacetic acid; benzylhydrazine; mechanism-based inhibition; substance P; neuropeptide

The bifunctional enzyme peptidylglycine α-amidating monooxygenase (PAM) is responsible for the post-translational activation of many peptide hormones from their glycine-extended precursors including substance P [1], which is involved in the generation of pain and inflammation. Inhibition of PAM can lead to a decreased steady-state level of substance P and a concomitant accumulation of its precursor, which has a C-terminal glycine [1]. Copper(II) and ascorbic acid are cofactors of peptidylglycine α-hydroxylating monooxygenase (PHM; EC 1.14.17.3), the first enzyme in the PAM reaction sequence [2]. PHM forms an α-hydroxyglycine intermediate, which in a second step, is cleaved by peptidylamidoglycolate lyase (PAL; EC 4.3.2.5) to generate the α-amidated peptide product and glyoxylate:



The copper-chelator disulfiram [1], as well as redox compounds such as sulfite ion [3] or benzylhydrazine [4], are inactivators of PHM. Most likely, the latter can form inactivating free radical intermediates by redox chemistry at the enzyme-associated Cu^{2+} ions, similar to ascorbic acid [3, 4]. Mechanism-based inactivation of PHM, most probably through free radical intermediates, has also been observed with olefinic substrate analogues [5, 6]. Approaches to the rational design of mechanism-based, irreversible olefinic inactivators of PHM have been reported by Vederas et al. [7] and Casara et al. [8].

Here, we report our results on inhibition of PHM from blood serum of different species and of PHM from different rat organs with *trans*-styrylacetic acid, *trans*-styrylthioacetic acid and benzylhydrazine. These compounds were previously shown to inactivate recombinant rat medullary thyroid carcinoma PHM secreted from Chinese hamster ovary cells [4], soluble PHM from bovine [5] or porcine [9] pituitary gland, or horse blood serum [8]. For concept

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Abbreviations: PAM, peptidylglycine α-amidating monooxygenase; PHM, peptidylglycine α-hydroxylating monooxygenase; PAL, peptidylamidoglycolate lyase.

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validation purposes, it appeared necessary to work with PHM prepared from tissues of ordinary laboratory animals. The present study was considered as a prerequisite to the search for an *in vivo* model permitting the pharmacological evaluation of such compounds on their potential disease target, e.g. inflammation. For this purpose, we applied a very sensitive and specific enzyme assay on rat tissues using the fluorescent substrate dansyl-Tyr-Val-Gly [10], which allowed the utilisation of a highly diluted, partially purified PHM preparation.

MATERIALS AND METHODS

Materials

The PAM substrate dansyl-Tyr-Val-Gly, bovine liver catalase (EC 1.11.1.6), 2-[N-morpholino] ethanesulfonic acid (MES), N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) and ascorbic acid were obtained from Sigma, St. Quentin Fallavier, France. D-Tyr-Val-Gly was purchased from Bachem-Feinchemikalien, Bubendorf, Switzerland, benzylhydrazine and 4-phenyl-3-butenoic acid (*trans*-styrylacetic acid) from Aldrich, St. Quentin Fallavier, France and Phenyl Sepharose 6 Fast Flow from Pharmacia Biotech, Orsay, France. The product of the PAM reaction, dansyl-Tyr-Val-NH_2, using solid-phase peptide synthesis, as well as *trans*-styrylthioacetic acid (acetic acid, [(2-phenylethenyl)thio]-, lithium salt, (E)-) [8] were prepared in our Strasbourg Research Center. The other chemicals and solvents were purchased from either Merck, Darmstadt, Germany, Fluka, Buchs, Switzerland, Carlo Erba, Val de Reuil, France or Aldrich, St. Quentin Fallavier, France. Adult male Sprague-Dawley rats were obtained from IFFA-Credo, France, and were kept under controlled animal house conditions, supplied with a standard laboratory diet.

Enzyme Preparation

A previously published procedure by May et al. [11] was adapted for the rat enzyme. Five to ten male adult Sprague–Dawley rats were killed by decapitation, the blood collected on ice and the tissues immediately frozen in liquid nitrogen. The serum was obtained after blood coagulation by centrifugation at 10000 × g. Tissues were homogenized in 9 volumes (w/v) of 20 mM MES buffer at pH 6.6. The homogenate was centrifuged for 5 min at 1000 × g in a cooled centrifuge followed by centrifugation of the supernatant at 100000 × g for 60 min. The final enzyme preparations were dialysed and stored at −80°C.

Soluble PHM

The enzyme was precipitated from the high-speed supernatant by stepwise addition of saturated ammonium sulfate solution over a period of 15 min to give a final ammonium sulfate concentration of 45%. Following another 15 min of stirring, the precipitate was collected by centrifugation at 27000 × g for 15 min and dissolved in 3 mL of buffer.

Particulate PHM

For the preparation of the particulate fraction, the homogenate was subjected to three freeze-thaw cycles prior to centrifugation. The high-speed centrifugation pellet was dispersed by sonication in 5 mL of 20 mM MES buffer at pH 6.6 containing 1% Tween 20 followed by stirring for 1 hr at 4°C. The supernatant was then isolated by centrifugation at 100000 × g for 60 min. The pellet from brain tissue was extracted twice. The collected extract was subjected to 55% ammonium sulfate precipitation with subsequent centrifugation at 100000 × g and dialysis of the redissolved pellet against buffer containing 0.05% Tween 20.

Serum PHM

For the preparation of PHM from blood serum, the ammonium sulfate-precipitate was discarded at 20% saturation. Following dialysis of the 45% precipitate, the dialysate was centrifuged at 100000 × g for 30 min to remove insoluble protein.

Horse and Human Serum PHM

After precipitation with 50% ammonium sulfate, the pellet was redissolved in 10 mM HEPES buffer, pH 7, containing 1 M ammonium sulfate. The solution was subjected to hydrophobic interaction chromatography on a 2.6 × 10 cm column packed with Phenyl Sepharose 6 Fast Flow (Pharmacia Biotech, Orsay, France). After washing with 10 mM HEPES at pH 7 containing 1 M ammonium sulfate, the PHM activity was eluted at a rate of 5 mL/min, using a 300 mL gradient of 1 M to 0 M ammonium sulfate in 10 mM HEPES at pH 7. The active fractions were concentrated to ca. 1 mL on a Centricon 30 filter (Amicon, Beverly, MA, USA). The specific activity of these partially purified preparations with Tyr-Val-Gly as substrate was approximately 30 nmol/hr/mg protein. The substrate K_M under standard assay conditions was 0.1 mM for both the horse and the human enzyme. PAL was completely eliminated during the purification.

Protein Determination

This was done according to the method published by Bradford [12].

Assay Methods

STANDARD **PHM** ASSAY. PHM activity was assayed according to Jones et al. [10]. Appropriate amounts of enzyme solution were incubated at 37°C, usually for 2 hr. The incubation mixture contained the following ingredients in a total volume of 500 μL of 0.1 M MES buffer at pH 6.6:

2 μM copper(II)-sulfate, 5 mM ascorbic acid, 6500 U catalase, 0.5 mM N-ethylmaleimide, 0.01% Tween 20 and 20 μM dansyl-Tyr-Val-Gly as substrate. The reaction was stopped on ice by addition of 18 μL of 35% perchloric acid followed by centrifugation. For the subsequent HPLC analysis of the reaction product dansyl-Tyr-Val-NH_2, 200 μL of the supernatant were mixed with 30 μL of 2 M potassium hydroxide/0.05 M EDTA to give a final pH of 8.5. Forty μL of this mixture were injected onto the HPLC column. Final enzyme activities were adjusted to obtain product formation in the range of 0.5–1.0 nmol/2hr/sample. Assays were run in duplicate and experiments were usually repeated at least once for confirmation.

Pre-incubation of PHM in the presence of potential inactivators was carried out under standard assay conditions, but in the absence of substrate. At the indicated incubation times, 20 μL samples were transferred to the final assay mixture which was kept on ice. Ascorbic acid was added shortly before assaying for residual enzyme activity. For the pre-incubation, the above assay conditions were changed with respect to the ascorbic acid and catalase concentration used: When the olefinic compounds were tested, the ascorbate concentration was reduced to 1 mM; ascorbate was not present when benzylhydrazine was used as the inactivator. In the latter case, the catalase content was also reduced to 500 U, because of a protective effect against benzylhydrazine-mediated inactivation [4].

PHM ASSAY USED WITH HORSE AND HUMAN BLOOD SERUM. Appropriate amounts of enzyme were incubated in 500 μL of 100 mM MES at pH 6.6, with 2 μM $CuSO_4$, 3 mM ascorbate, 6500 U of catalase and 0.2 mM Tyr-Val-Gly as substrate. The reaction was stopped after 90 min by adding 20 μL of 2 M perchloric acid to 200 μL assay mixture and analysed by HPLC. Pre-incubations in the presence of inhibitors were carried out under identical conditions in the absence of substrate. Aliquots were removed at given time points and diluted 20-fold into the assay mixture, containing 1 mM of Tyr-Val-Gly, to determine residual enzyme activity.

HPLC Analysis

DANSYL-TYR-VAL-NH_2. A Superspher RP 18 column (150 × 4 mm i.d., 4 μm particle size) (Bischoff, Leonberg, Germany) was used. Isocratic elution was performed with 0.1 mM sodium acetate buffer at pH 6.5 containing 44% acetonitrile at a rate of 1 mL/min. The duration of a run following automatic injection was set at 5 min. Fluorescence detection was used at an excitation wavelength of 356 nm and an emission wavelength of 482 nm. The peak which eluted after ca. 3 min was identified and quantitated using an external standard of 0.5 nmol dansyl-Tyr-Val-NH_2 per 0.5 mL.

TABLE 1. Kinetic properties of PHM preparations from different rat organs using dansyl-Tyr-Val-Gly as substrate

Enzyme	K_M μM	V_{max} nmol/hr/mg protein
Rat brain (soluble)	4 ± 1	86 ± 8
Rat brain (membrane-assoc.)	6 ± 2	34 ± 5
Rat heart atrium (membrane-assoc.)	9 ± 4	900 ± 220
Rat blood serum (soluble)	4 ± 1	9 ± 1

Values represent means of experiments run in triplicate ± SE.

TYR-VAL-GLY-OH. In the absence of PAL and under slightly acidic assay conditions, the product of the PHM reaction, Tyr-Val-Gly-OH, is sufficiently stable to be analysed by HPLC [13]. In assays with the horse and human serum enzymes, Tyr-Val-Gly-OH was separated from the substrate Tyr-Val-Gly on a Nova-Pak C_{18}-column (150 × 4 mm i.d., 4 μm particle size) (Millipore, St. Quentin-Yvelines, France) using a 10 mL gradient of 8 to 40% acetonitrile/water with 0.04% phosphoric acid at a rate of 1 mL/min. Spectrophotometric detection at 280 nm was applied. Tyr-Val-Gly-OH, as characterised by LC-MS, eluted at 5.4 min. No Tyr-Val-NH_2 was detected under these conditions.

RESULTS

Characterisation of Rat Enzymes

The PHM assay using dansyl-Tyr-Val-Gly as the substrate followed a linear time-course for at least 2 hr (not shown). The presence of Cu^{2+} and ascorbic acid in the incubation mixture was essential in all enzyme preparations. This fact unambiguously characterised the measured activities as PHM. The different rat PHM preparations did not always contain PAL in sufficient amounts to catalyse the complete breakdown of the intermediate α-hydroxyglycine derivative to the final product. PAL can be proteolytically split off the integral PAM molecule under physiological conditions [14]. Therefore, samples were generally alkalinised to pH 8.5 before their quantitative chromatographic evaluation, which ensured the complete non-enzymatic conversion of the hydroxylated intermediate to the final α-amidated reaction product [13].

Using standard conditions of pH as well as Cu^{2+} and ascorbate concentrations, the K_M- and V_{max}-values of the brain and serum PHM closely resembled each other (Table 1), whereas the membrane-associated PHM from heart atrium showed a slightly elevated K_M and an at least 10-fold higher specific activity in the crude enzyme preparation as compared to the other tissues (Table 1), in agreement with previously reported findings [15].

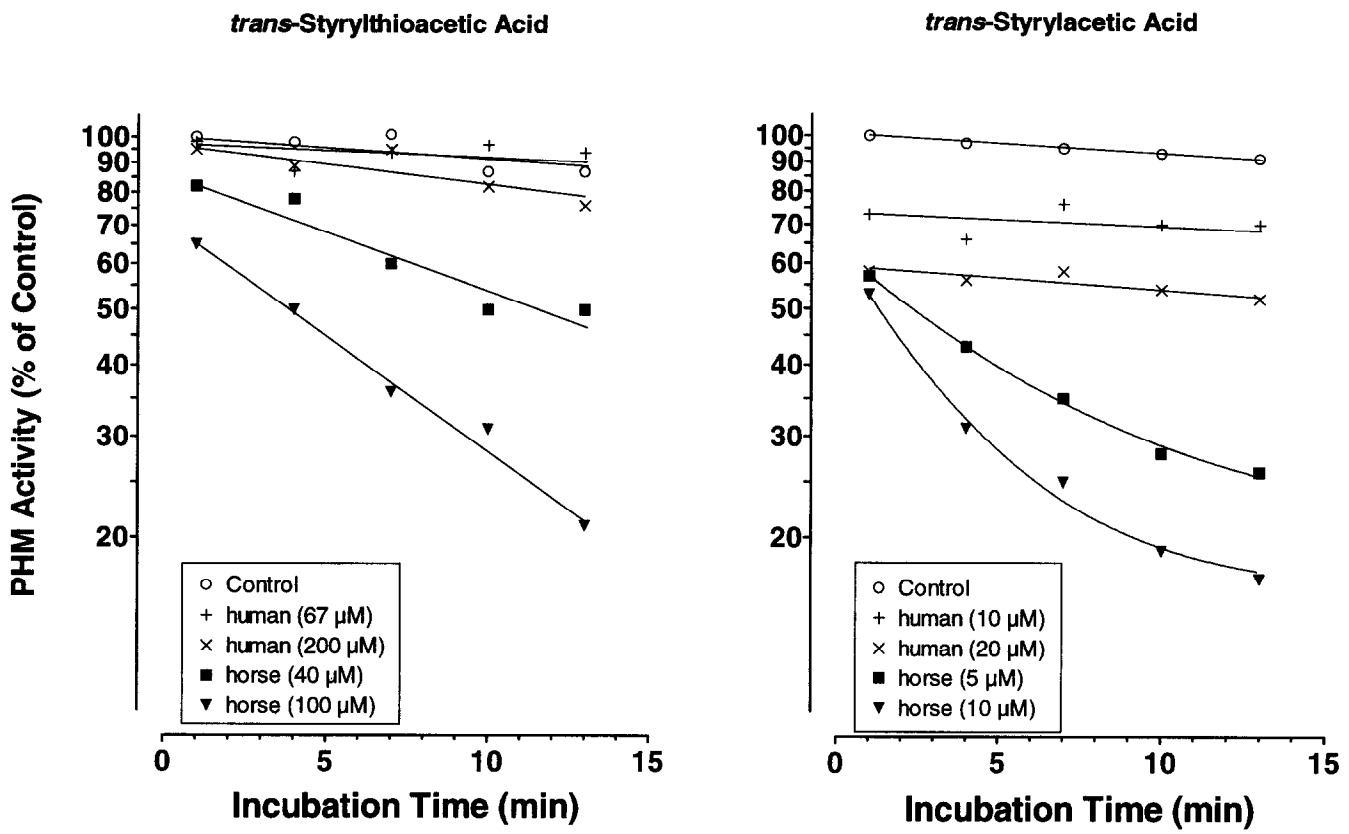

FIG. 1. Inactivation of human and horse serum PHM with *trans*-styrylacetic or *trans*-styrylthioacetic acid. The enzymes were pre-incubated with inhibitor in the presence of cofactors. At the indicated time points, aliquots were diluted for the assay of the residual PHM activity, using the substrate Tyr-Val-Gly. The product Tyr-Val-Gly-OH was determined by HPLC.

Inactivation Experiments

INTERSPECIES VARIATIONS OF SERUM PHM INACTIVATION USING TRANS-STYRYLACETIC OR TRANS-STYRYLTHIOACETIC ACID. Inactivation experiments were carried out over a pre-incubation period of 15 min with subsequent dilution of the pre-incubated enzyme into the assay mixture. Horse serum was described as a rich source of PHM and especially well-suited for purification of the enzyme [13]. Unsaturated thioacetic acids have been characterised as mechanism-based, irreversible inactivators of horse serum PHM [8]. During the course of our experiments, however, it became clear that the PHM preparations from horse serum reacted much faster than the human enzyme (Fig. 1). Whereas more than 50% of the activity of the horse serum PHM was lost within 5 min at 10 μM *trans*-styrylacetic acid, the human enzyme showed no time-dependent effect at the same concentration (Fig. 1). A more precise analysis of the data was complicated by non-linear inactivation kinetics in accordance with a previous report [9].

A similar species selectivity was observed with *trans*-styrylthioacetic acid (Fig. 1); however, the time-dependent inhibition in this case more closely followed pseudo-first order kinetics, although occurring at a slower initial rate. Thus, for the horse serum PHM, a $k_{inact}/K_I \approx 35\ M^{-1}\ sec^{-1}$ [8] could be determined, whereas barely detectable inhibition was found with the corresponding human enzyme (Fig. 1).

INACTIVATION OF PHM PREPARATIONS FROM DIFFERENT RAT ORGANS USING TRANS-STYRYLACETIC OR TRANS-STYRYLTHIOACETIC ACID. The rat was chosen for further inhibition studies. Time-dependent inactivation of the rat serum PHM with *trans*-styrylacetic acid was observed (Fig. 2), occurring at an apparently slower rate than with horse serum. The presence of ascorbate was essential for this inactivation (not shown). Again, the inactivation curves followed non-linear kinetics. *Trans*-styrylacetic acid-mediated inactivation was also found with the membrane-associated PHM from rat heart atrium. Rough estimates of the kinetic constants were obtained from the non-linear plots using the initial inactivation rates. The kinetic constants (Table 2) were calculated using a Kitz and Wilson plot [16], which was linear and did not pass through the origin (not shown). Using rat serum PHM under the same reaction conditions, *trans*-styrylacetic acid was a more potent inhibitor than *trans*-styrylthioacetic acid (Fig. 2), which, again, more closely followed pseudo-first order kinetics. In contrast, with the soluble brain PHM, time-dependent inactivation was not detectable under our reaction conditions (Fig. 3), even at a maximal concentration of 100 μM *trans*-styrylacetic acid (not shown). A similar result was obtained with the membrane-associated rat brain PHM (not shown). In order to exclude the possibility that a component of the brain extract might have an effect, we mixed the membrane-associated brain PHM with the serum

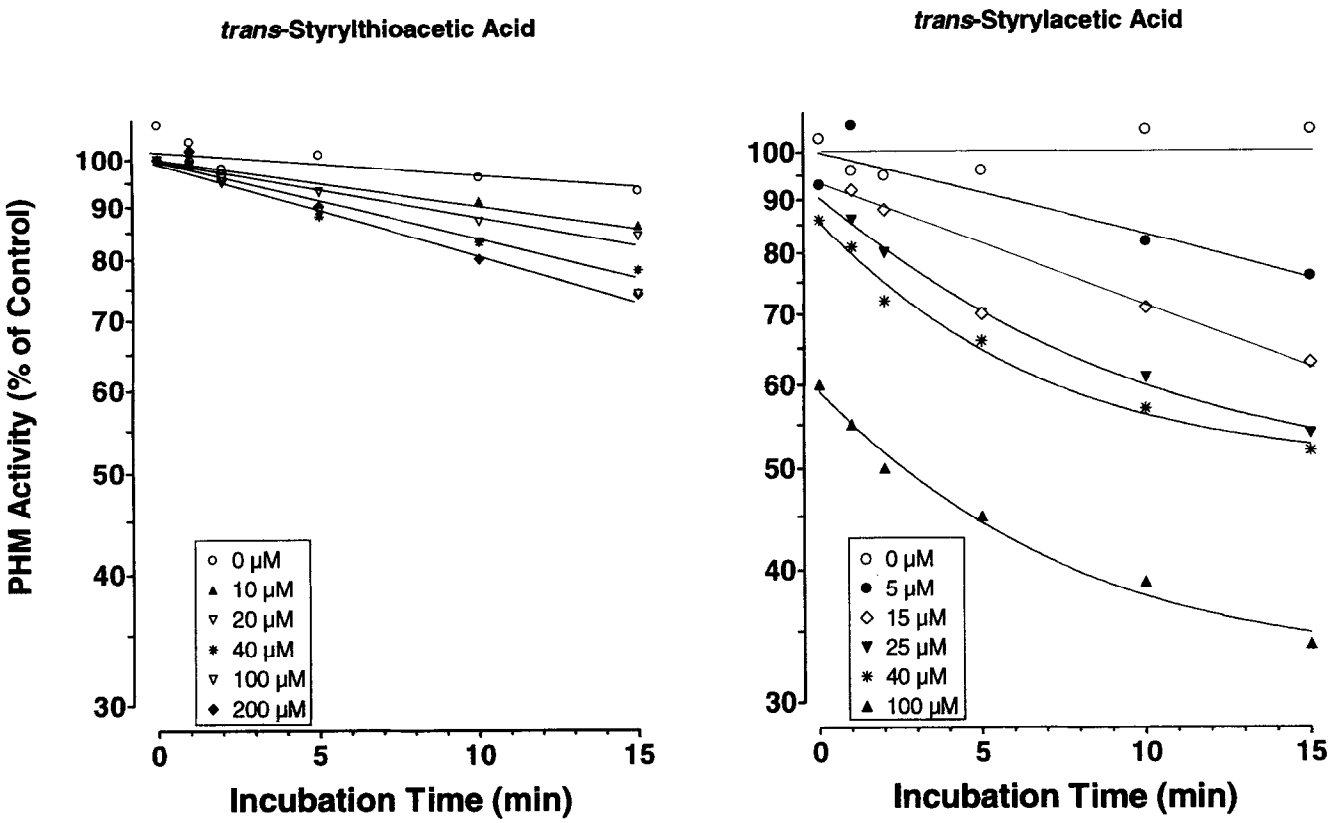

FIG. 2. Comparative inactivation of rat serum PHM with either *trans*-styrylacetic or *trans*-styrylthioacetic acid. The experiment was performed as in Fig. 1, except that dansyl-Tyr-Val-Gly was used as the substrate for the assay of the residual PHM activity. Duplicate samples were run under standard conditions; the data of this experiment in combination with two other experiments were used for the kinetic data shown in Table 2.

enzyme, thereby demonstrating that this did not affect the expected amount of the trans-styrylacetic acid-mediated inactivation of the serum PHM (Table 3) and again confirming the absence of a time-dependent effect on the brain enzyme.

Using *trans*-styrylacetic acid, a reversible inhibitory component was observed at time zero of the pre-incubation (Fig. 1 and 2), suggesting a high affinity of the compound for the enzyme. With rat brain a similar high affinity towards PHM was found, whereas no time-dependent

TABLE 2. Kinetic constants for time-dependent inactivation of PHM preparations from different rat organs using *trans*-styrylacetic acid

Enzyme	k (min^{-1})	K_I (μM)	k_{inact}/K_I (M^{-1} sec^{-1})
Rat brain (soluble)	no time-dependent inhibition		
Rat brain (membrane-associated)	no time-dependent inhibition		
Rat heart atrium (membrane-associated)	0.29 ± 0.04	47 ± 1	103 ± 8
Rat blood serum (soluble)	0.26 ± 0.05	42 ± 14	102 ± 7

Values (± SE) were calculated by Michaelis–Menten-like non-linear regression using the computer program Enzfitter version 1.04 M.

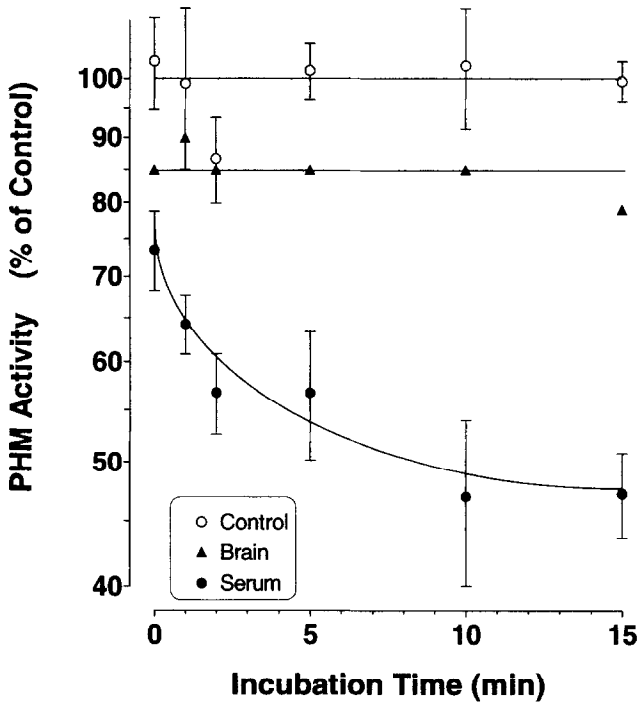

FIG. 3. Inactivation of rat serum and soluble rat brain PHM with 50 μM *trans*-styrylacetic acid. Standard conditions were used. Values represent means of 6 controls and 4 inactivation experiments for the serum preparation ± SD. For the brain preparation, only one experiment at this concentration is shown; however, absence of reactivity was also observed at 100 μM.

TABLE 3. Absence of an effect of membrane-associated rat brain PHM on *trans*-styrylacetic acid-mediated inactivation of rat serum PHM

Incubation Time (min)	Absence of *trans*-Styrylacetic Acid			Presence of 50 μM *trans*-Styrylacetic Acid		
	Brain[a]	Brain/Serum[b] (50:50)	Serum[c]	Brain	Brain/Serum (50:50)	Serum
0	100	100	100	100	100	100
1	103	93	99	97	98	89
2	103	99	98	94	92	83
5	101	95	95	92	82	69
10	102	100	94	92	81	62
15	103	98	90	90	77	53

Means of duplicate determinations; λ for a better comparison in this case, residual enzyme activities are expressed as % of the values at time zero, which does not reflect the competitive inhibitory component observed with *trans*-styrylacetic acid.

[a] Mean activity = 0.77 nmol product formed/2 hr per sample; [b]mean activity = 0.57 nmol product formed/2 hr per sample; [c]mean activity = 0.35 nmol product formed/2 hr per sample.

inactivation occurred. In this case, the reversible K_I-values could be determined, as exemplified in Fig. 4 by the Dixon plot for the membrane-associated PHM. The data in Fig. 4 were analysed using the computer programs COMP for competitive and NONCOMP for noncompetitive inhibition [17]. A good fit was obtained with the competitive model providing $K_I = 1.0 \pm 0.1$ μM. In contrast, treatment of the data using the NONCOMP algorithm did not result in a reasonable fit. This indicates that there are no significant intercept effects in 1/v versus 1/S plots, and that the data are compatible with competitive inhibition by *trans*-styrylacetic acid versus the peptide substrate. Using the soluble PHM, a competitive $K_I = 0.6 \pm 0.15$ μM was obtained, whereas again the data did not fit with the noncompetitive model. The reversible, competitive inhibition constants clearly represent affinities for the substrate-binding site, whereas the constants obtained for time-dependent inhibition (Table 2) comprise the partition ratios of the reactive intermediates as well. A great difference between these values is thus observed.

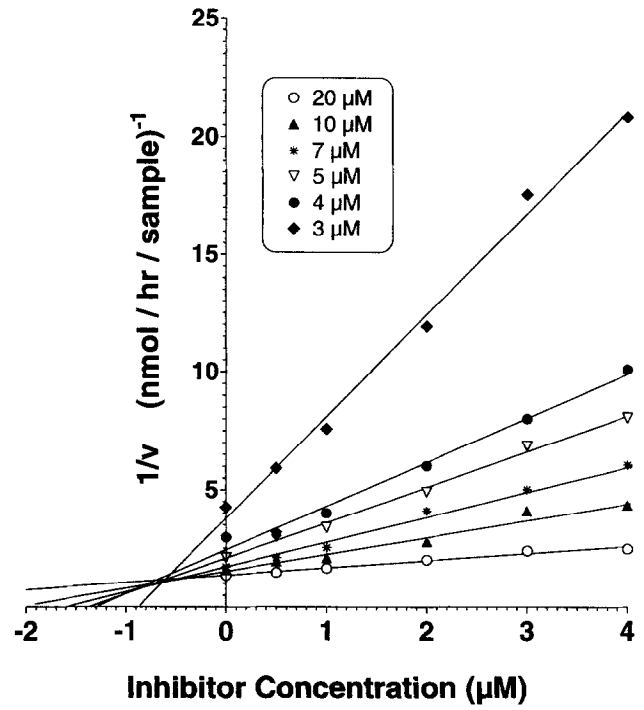

FIG. 4. Reversible inhibition of particulate rat brain PHM with *trans*-styrylacetic acid. Duplicate samples were run under standard conditions with dansyl-Tyr-Val-Gly as substrate and 1 hr incubation time. The substrate concentrations are given in the legend.

INACTIVATION OF DIFFERENT PHM PREPARATIONS USING BENZYLHYDRAZINE. Benzylhydrazine at 12 mM inactivated the rat serum PHM by approximately 80% during the 15-min pre-incubation period (Fig. 5), following pseudo-first order kinetics. The membrane-associated enzyme from rat heart atrium was similarly inactivated (not shown). The data did not yield a linear Kitz and Wilson plot (Figure 5, Insert), in agreement with a previous study [4] where the inactivation was found to be mechanism-based, which we could confirm by its Cu^{2+}-dependence (not shown). In agreement with our findings using *trans*-styrylacetic acid, the soluble (Fig. 5) as well as the membrane-associated PHM (not shown), isolated from rat brain, were completely inert against 12 mM benzylhydrazine over the pre-incubation period.

DISCUSSION

Previous studies using alkenoic acids for PHM inactivation [5, 9], involved partially purified enzyme preparations from porcine or bovine pituitary. The fact that the enzyme was not pure did not appear to have an effect on the validity of the results [6]. Moreover, the different partially purified PHM preparations from rat tissues used in the present study resembled each other with respect to their affinity for the substrate as well as their requirement for Cu^{2+} and ascorbic acid. Thus, mechanism-based inactivation of PHM from blood serum as well as the membrane fraction of rat heart atrium, in the presence of alkenoic acid derivatives or benzylhydrazine, was also found in the present study. Such inactivation by olefinic compounds is explained by the

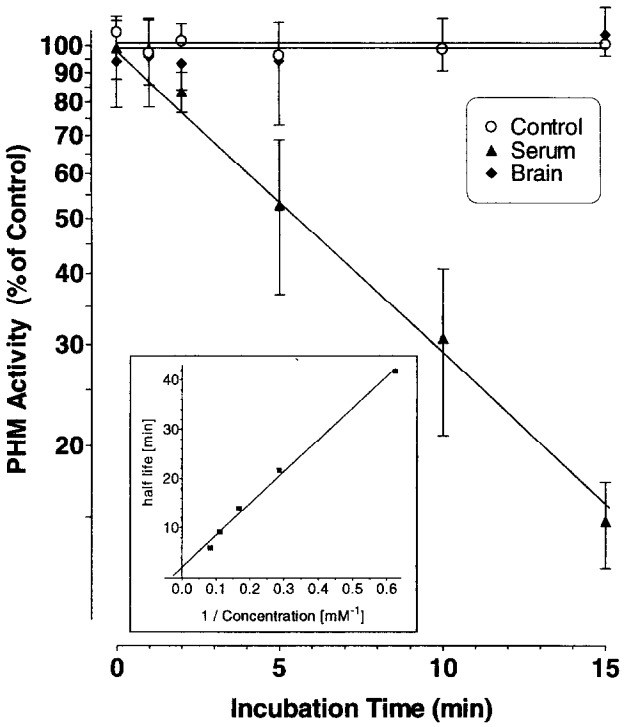

FIG. 5. Inactivation of rat serum or soluble rat brain PHM with 12 mM benzylhydrazine. Standard conditions were used. Values represent means of 4 to 12 experiments ± SD. Insert: Kitz and Wilson plot for benzylhydrazine-mediated inactivation of rat serum PHM. Values represent means of duplicate determinations.

close analogy to the mechanism postulated for dopamine β-monooxygenase (EC 1.14.17.1) inactivators [18]. *Trans*-styrylacetic acid or *trans*-styrylthioacetic acid probably react with PHM by forming a radical intermediate that in turn can attach to an amino acid residue within the enzyme's active center [6, 8]. Being short-chain analogues of the natural glycine-extended peptide substrates of PHM, these "suicide-inhibitors" were expected to bind to the substrate-binding site. This assumption is supported by the reversible, competitive inhibition of the rat brain PHM by *trans*-styrylacetic acid, as observed in the present study. The substrate-like behaviour was also confirmed by the ascorbic acid-dependence of serum PHM-inactivation.

The up-to-now unreported fact that the brain PHM was not inactivated, although strong substrate-like binding occurred, suggests mechanistic differences. The olefin-modifiable amino acid residues could be, for example, some essential histidine groups postulated to co-ordinate the active-site copper atoms [19]. These histidines are conserved among PHM isolated from rat, human, cattle and frog [20], and their site-directed mutagenesis can result in an inactive enzyme species [19, 20]. Brain PHM catalysis may not involve a free radical sufficiently reactive towards such essential amino acid residues, thus accounting for the different susceptibility to inactivation. However, there is no obvious reason to assume such mechanistic differences, given the similarity between the K_M-values of the PHMs isolated from rat. Therefore, as an alternative for the serum and heart atrium enzymes, it appears more likely that covalent attachment of a free radical intermediate occurs at a non-essential amino acid residue, preventing the subsequent substrate access. Polymorphism of the active-site residues among the different PHM isozymes may thus lead to an altered partition ratio for a mechanism-based inactivator. The lack of susceptibility to inactivation of the rat brain PHM was corroborated using benzylhydrazine, which upon processing by PHM is believed to form a benzyl radical-intermediate [4]. In the case of serum or heart atrium PHM, both radical intermediates formed from either *trans*-styrylacetic acid or benzylhydrazine may thus react with the same active-site residue, being out of reach in the brain enzyme. In this context we suggest, in addition to the extensively studied complex posttranscriptional [2] and -translational [21, 22] modifications of the PAM molecule, an investigation into the tertiary structures of the isozymes, which would enhance our knowledge of the active-site topology and thus the biochemical behaviour of PHM.

The above findings also imply the usefulness of *trans*-styrylacetic acid or benzylhydrazine as tools for selective inactivation of peripheral PHM isozymes versus brain isozymes *in vivo*. Since PAM-mediated activation is essential for substance P and its function in pain threshold [1], an inhibitor of its formation could be useful in the treatment of rheumatoid arthritis. However, a number of other important hormonal peptides are also α-amidated by PAM [23], e.g. in the pituitary gland. More detailed information on the tissue distribution of PHM isozymes as well as potential inactivators are thus needed for the specific control of a distinct PAM-dependent hormonal function.

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