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ACTIVATION OF AN ADRENERGIC PRO-DRUG THROUGH SEQUENTIAL STEREOSELECTIVE ACTION OF TANDEM TARGET ENZYMES

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SUMMARY: The synthetic amino acid, 3,4-dihydroxyphenylserine (DOPS) has been of great interest for many years as an adrenergic pro-drug, since the L-threo diastereomer of DOPS can be a precursor of R-(-)-norepinephrine, the natural form of this neurotransmitter. We now report bioactivation of DOPS to the potent pharmacological agent, noradrenalone (arterenone), via sequential stereoselective action by two target enzymes -- dopamine β-monooxygenase (DBM) and L-aromatic amino acid decarboxylase (AADC) -- acting in tandem. Enzymatic activation is stereospecific, with only the L-erythro DOPS diastereomer producing noradrenalone; this is consistent with the known stereospecificities of AADC and DBM. These results provide a heretofore unrecognized rationale for the bioactivity of L-erythro DOPS and provide a basis for the design of new adrenergic pro-drugs.

The synthetic amino acid, 3,4-dihydroxyphenylserine (DOPS) has been of great interest for many years as a potential adrenergic pro-drug (1-9). DOPS exists as four stereoisomers -- (+)-L-erythro, (-)-D-erythro, (-)-L-threo, and (+)-D-threo -- due to the chirality of both the amino acid (α-carbon) and carbinol (β-carbon) moieties. It has long been recognized that L-threo-DOPS (αS, βR) can be converted in many tissues to (-)-norepinephrine (NE), which is the natural form of this primary adrenergic neurotransmitter. Thus, the use of DOPS as an adrenergic pro-drug has been proposed for hypotensive disorders (7), for depression (8), and for Alzheimer's and Parkinson's diseases (8), and a successful clinical study has been carried out in which DOPS was administered as a therapeutic bypass for a catecholamine enzyme deficiency (9).

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In contrast to the L-threo diastereomer, enzymatic decarboxylation of L-*erythro*-DOPS (αS, βS) would produce the "unnatural" (+)-enantiomer of norepinephrine, whose βS configuration is *opposite* to the βR configuration present in all of the functional phenylalkanolamine neurotransmitters and hormones (i.e., NE, epinephrine and their cognates). We have previously demonstrated that the enzyme dopamine β-monooxygenase (DBM, EC 1.14.17.1) readily catalyzes ketonization of (S)-β-phenylethanolamines such as (S)-octopamine, norpseudoephedrine, and (S)-norepinephrine, producing the corresponding ketones via stereospecific (R)-hydroxylation at the β-carbon followed by dehydration of the enzymatically-produced *gem*-diols (10-12). Thus, we reasoned that sequential stereoselective action of L-aromatic amino acid decarboxylase (AADC; EC 4.1.1.38) and DBM on L-erythro-DOPS should result in production of noradrenalone (arterenone) -- the ketone analog of norepinephrine -- which is a highly potent pharmacological agent (13-15). Such a process would represent an example of production of a desired pharmacologically-active compound from an inactive precursor via sequential stereoselective action by two target enzymes acting in tandem.

In this report we demonstrate stereoselective activation of the adrenergic pro-drug, DOPS, through tandem action of these two target enzymes.

EXPERIMENTAL

Dopamine β monooxygenase was isolated and purified from bovine adrenal glands as described previously (10,11). L-aromatic amino acid decarboxylase was obtained from hog kidney by ammonium sulfate fractionation using the method of Voltattorni et. al.(16). Noradrenalcne was synthesized as described by Remizov (17) and recrystallized from ethanol/ether as the hydrochloride salt; mp. 133-137(d); Mass spec (CI): m/e 138.1 (M+1); ^{1}H nmr (δ,D2O): 7.45-6.85 (m,3H), 4.43 (s,2H). The four diastereomers of 3,4-dihyroxyphenylserine were generously supplied by Sumitomo Pharmaceuticals Co., Ltd. (Osaka, Japan), and purity was confirmed by hplc analysis. L-threo: mp 230(d); $[\alpha]_D^{20}$, -39.2. D-Threo: mp 230(d); $[\alpha]_D^{20}$, +42.4. L-erythro: mp 162(d); $[\alpha]_D^{20}$, +49.2. D-erythro: mp 153-156(d); $[\alpha]_D^{20}$, -59.5. All other chemicals and solvents were obtained from standard chemical sources and were of highest purity.

L-aromatic amino acid decarboxylase reactions were carried out at 37 °C in a medium containing 100 mM phosphate buffer (pH 6.8), 20 μM pargyline, 10 μM pyridoxal phosphate, and 15 mM of the particular DOPS isomer. After a 5 minute preincubation of the medium (0.45 ml), reaction was initiated with 0.15 ml of enzyme solution (ca. 2.5 mg protein). Aliquots for HPLC analyses were

taken at various times and quenched with an equal volume of ice-cold 0.4 N perchloric acid containing 2.5 mg/ml sodium metabisulfate and 50 mg/ml EDTA, and then centrifuged at 5000 g for 10 minutes. For coupled assays with dopamine β-monooxygenase, 0.5 ml of the decarboxylase assay mixture from above was combined with 0.45 ml of a solution of 20 mM ascorbic acid, 20 mM sodium fumarate, 10 μM $CuSO_4$, 500 μg/ml catalase in 0.1 M sodium acetate buffer (pH 5.25). After preincubated for five minutes, the reaction was initiated with 0.1 ml of a DBM solution (ca. 1 μg protein). Aliquots for HPLC analysis were quenched as described above.

HPLC analyses were performed using a dual electrode electrochemical detection system equipped to oxidize catecholamines (ESA 5100A, Analytical Cell 5011 with cell 1 set at -100 mV, cell 2 set at +400mV, and the conditioning cell 5021 set at -250mV), equipped with a dual piston pump (ESA Model 420), and strip recorder (Fisher 500). Products were separated utilizing an ESA Catecholamine HR-80 column (8cm X 4.6mmID, 3 micron ODS) and a mobile phase of 6.9 g/L sodium phosphate monobasic, 250 mg/L 1-heptanesulfonic acid (Na salt), 80 mg/L EDTA, and 5% methanol, adjusted to pH 3.6 with NaOH.

RESULTS AND DISCUSSION

In separate experiments, each of the four diastereomers of 3,4-dihydroxyphenylserine was incubated with L-aromatic amino acid decarboxylase (AADC) and then with dopamine β-monooxygenase (DBM). The results obtained are presented in Table 1. As expected, L-threo- and L-erythro- DOPS, both of which possess the (S) configuration at the α-carbon, are active substrates for AADC. In

Table 1. Reactivities of DOPS Diastereomers in Coupled Assays

Isomer	Stereochemistry	Activity with AADC[a]	Activity with DBM[b]
L-erythro	(αS,βS)	+	+
L-threo	(αS,βR)	+	-
D-threo	(αR,βS)	-	-
D-erythro	(αR,βR)	-	-

[a] Measured by the production of norepinephrine, as determined by HPLC analysis, after incubation with AADC for 60 minutes.

[b] Measured by the production of noradrenalone, as determined by HPLC analysis, after sequential incubation with AADC for 90 minutes, followed by DBM for 60 minutes.

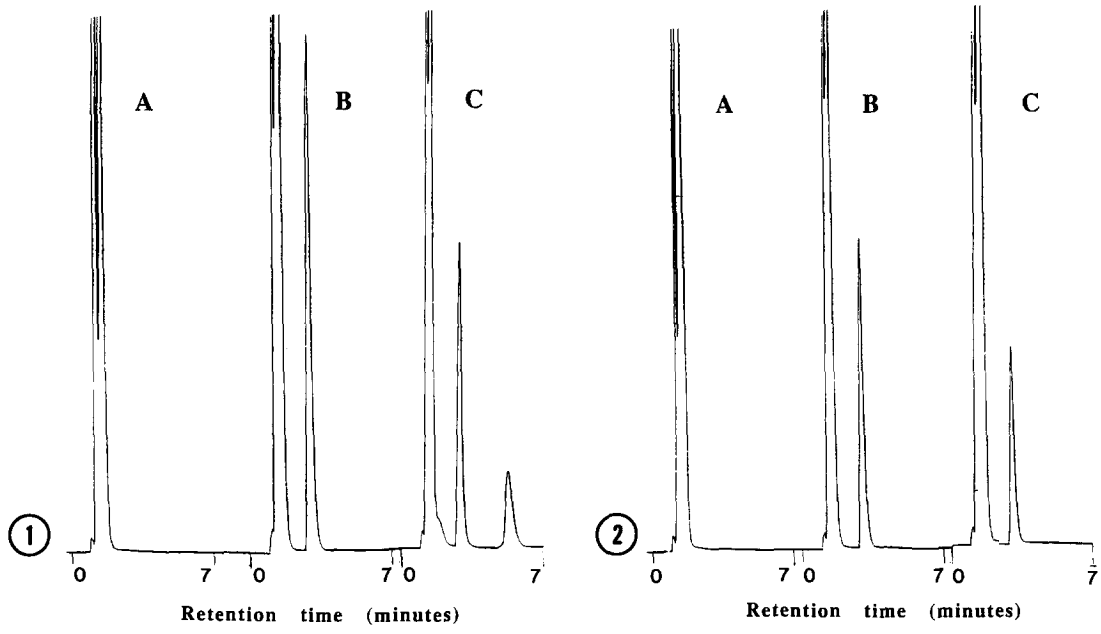

Figure 1. Identification of products by HPLC of L-erythro-DOPS from coupled assay. A) Chromatrogram of assay medium prior to incubation with enzymes. The large peak consists primarily of L-erythro-DOPS. B) Chromatogram after incubation with AADC for 60 minutes. The new peak with an elution time of 2.5 minutes was identified as norepinephrine by comparison with an authenic standard. C) Chromatogram of assay medium after incubation with DBM for 60 minutes, preceded by AADC incubation for 90 minutes. The third peak at 4 minutes co-elutes with an authentic standard of noradrenalone.

Figure 2. Identification of products by HPLC of L-threo-DOPS from coupled assay. A) Chromatrogram of assay medium prior to incubation with enzymes. The large peak consists primarily of L-threo-DOPS. B) Chromatogram after incubation with AADC for 60 minutes. The new peak with an elution time of 2.5 minutes is norepinephrine. C) Chromatogram of assay medium after incubation with DBM for 60 minutes, preceded by AADC incubation for 90 minutes. A noradrenalone peak does not appear. The reduction in height of the norepinephrine peak is due to dilution of concentrations upon addition of medium for the DBM reaction.

contrast, both D-threo- and D-erythro- DOPS, possessing the (R) configuration at their α-carbon atoms, are inactive toward AADC. It is also evident from the Table that although both "L" diastereomers are AADC substrates, only L-erythro-DOPS -- and not L-threo-DOPS -- is converted by AADC to a product which undergoes a subsequent reaction with DBM.

Figures 1 and 2 illustrate HPLC chromatograms obtained after reaction of DOPS diastereomers with AADC and DBM. As shown in the figures, reaction of AADC with either L-erythro- or L-threo-DOPS results in time-dependent formation of norepinephrine (NE), the expected product of enzymatic decarboxylation. Control

experiments confirmed that NE formation does not occur when either D-threo- or D-erythro- DOPS is incubated with AADC, and that non-enzymatic decarboxylation does not occur with any of the DOPS diastereomers under the reaction conditions.

While AADC-catalyzed decarboxylation clearly produces NE from both L-erythro- and L-threo-DOPS, the products arising from these two substrates are enantiomeric, with L-threo-DOPS producing R-(-)-NE and L-erythro-DOPS producing S-(+)-NE. Since we have previously established that DBM stereospecifically catalyzes ketonization of *only* (S)-β-phenylethanolamines (10-12), our expectation was that only the product produced by AADC from the L-erythro-diastereomer of DOPS would undergo DBM-catalyzed ketonization. It is clear from Figures 1C and 2C that this is indeed the case. As shown in Figure 1C, sequential reaction of L-erythro-DOPS with AADC and DBM results in formation of noradrenalone in a time dependent process. As expected, adrenalone formation from the L-erythro substrate does not occur when either enzyme is omitted from the experiment. In sharp contrast, Figure 2C shows that noradrenalone formation does <u>not</u> occur when L-threo-DOPS is reacted sequentially with AADC and DBM.

Taken together, these results establish that activation of DOPS through sequential action by AADC and DBH indeed occurs with the stereochemistry expected on the basis of the respective stereospecificities of AADC (3) and DBM (10). Thus, of the four stereoisomers of DOPS, <u>only</u> the L-erythro diastereomer is capable of being enzymatically converted to the potent pharmacological agent, noradrenalone, through the tandem action of these two target enzymes.

Kinetic experiments were carried out with the AADC-reactive DOPS diastereomers. K_m values of 2.81 mM and 2.39 mM were obtained for L-erythro-DOPS and L-threo-DOPS, respectively. While these K_m values are similar, the V_{max} values of 6.6×10^{-4} mM/s and 6.4×10^{-5} mM/s obtained under our assay conditions for L-erythro-DOPS and L-threo-DOPS, respectively, differ markedly. Thus, it is evident from these kinetic results that L-erythro-DOPS -- the only diastereomer which can undergo the bioactivation to noradrenalone which we report here -- is indeed the more reactive diastereomer.

The possibility of developing specific, enzyme-targeted molecules of potential clinical interest is the primary goal of much current effort directed toward rational design of enzyme-activatable "pro-drugs". The usual design strategy focuses on a specific target enzyme capable of converting the pro-drug to a

pharmacologically-active species. The results reported here illustrate an example of "pre-pro drug" activation, whereby the desired pharmacologically-active compound is produced from an inactive precursor via sequential stereoselective action by two target enzymes acting in tandem.

Over the years, many investigators have reported finding unexpected bioactivity for the L-erythro diastereomer of DOPS (18). These observations have been puzzling since metabolic decarboxylation of L-erythro-DOPS should give rise to "unnatural" (+)-norepinephrine, which is receptor-inactive. Our demonstration that L-erythro-DOPS undergoes the sequential enzymatic biactivation process reported here to produce noradrenalone provides an attractive rationale which may account for the results obtained in previous studies, and may provide a basis for the design of future adrenergic pro-drugs.

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