Biochimica et Biophysica Acta, 568 (1979) 339—347

BBA 68748

NUCLEOSIDE DEOXYRIBOSYLTRANSFERASE-II FROM *LACTOBACILLUS HELVETICUS*

SUBSTRATE SPECIFICITY STUDIES. PYRIMIDINE BASES AS ACCEPTORS

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(Received November 27th, 1978)

Key words: Nucleoside-deoxyribosyltransferase II; Substrate specificity; Pyrimidine analog (Lactobacillus)

Summary

The nucleoside deoxyribosyltransferase (nucleoside:purine (pyrimidine) deoxyribosyltransferase, EC 2.4.2.6) fraction catalyzing specifically the transfer of the deoxyribosyl moiety from a purine (or a pyrimidine) to a pyrimidine (or a purine) exhibits a broad specificity for the acceptor base. With a pyrimidine base as the acceptor a -OH or -SH group adjacent to the N-1 atom is essential. A substituent on position 6 hinders the reaction. On positions 4 and 5 various substituents were found to influence the reaction rate and some of them give non-competent substrates. A few anomalous cases are also discussed in relation with the role of N-3. Deoxyribonucleosides can also be obtained with non-pyrimidine rings.

Introduction

In a few species of the Lactobacillus genus a direct transfer of the deoxyribosyl moiety between purine or pyrimidine bases is catalyzed by a nucleoside deoxyribosyltransferase (nucleoside:purine (pyrimidine) deoxyribosyltransferase, EC 2.4.2.6) [1]. This is a rare example of a direct deoxyribosyl transfer and is generally considered as an essential step in the salvage pathway for

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Abbreviations: Pur, a purine base; Pyr, a pyrimidine base; Pur ⇌ Pyr, a transfer reaction where the deoxyribosyl moiety is transferred from a purine to a pyrimidine; deoxynucleosides are abbreviated as recommended by IUPAC-IUB; dIno → Ade, a transfer in which deoxyinosine and adenine are the substrates.

bacteria which do not contain the nucleoside phosphorylases. These bacteria require vitamin B-12 [2] as precursor of the co-factor participating in the reaction catalyzed by the ribonucleotide triphosphate reductase. In the absence of vitamin B-12 an alternate pathway is the formation of the necessary deoxyribonucleotides from a pool of bases and some required deoxyribonucleoside.

In *Lactobacillus helveticus* three types of transfer can be observed:

$$\text{dRib-Pur} + \text{Pur}' \rightleftharpoons \text{dRib-Pur}' + \text{Pur} \quad (\text{Pur} \rightleftharpoons \text{Pur})$$

$$\text{dRib-Pur} + \text{Pyr} \rightleftharpoons \text{dRib-Pyr} + \text{Pur} \quad (\text{Pur} \rightleftharpoons \text{Pyr})$$

$$\text{dRib-Pyr} + \text{Pyr}' \rightleftharpoons \text{dRib-Pyr}' + \text{Pyr} \quad (\text{Pyr} \rightleftharpoons \text{Pyr})$$

From a preliminary Tris inhibition study Roush and Betz [3] concluded that two kinds of nucleoside deoxyribosyltransferase were probably present in *L. helveticus* and recently [4] it was possible to isolate two distinct fractions: one responsible for ($\text{Pur} \rightleftharpoons \text{Pur}$) transfer exclusively (nucleoside deoxyribosyltransferase-I) and the other catalyzing ($\text{Pur} \rightleftharpoons \text{Pyr}$) and ($\text{Pyr} \rightleftharpoons \text{Pyr}$) transfers as well (nucleoside deoxyribosyltransferase-II). A number of studies [1,3,5—11] have indicated that nucleoside deoxyribosyltransferases exhibit a rather broad specificity for the acceptor base and more recently we gave some of the structural conditions required for a purine base to be a substrate for nucleoside deoxyribosyltransferase-I [12]. Nucleoside deoxyribosyltransferase-II is therefore of great practical interest since it provides a simple and rapid means to prepare a number of deoxyribonucleoside analogues in amounts usually sufficient to determine their essential physicochemical and biological properties and particularly their cytotoxic character. Such a series of compounds may also be used in the study of bacterial deoxynucleoside-transporting systems and particularly the deoxynucleoside binding sites located on the surface of the bacterial cell [13].

Earlier studies showed that the natural pyrimidine bases and a few analogues are competent acceptors. However, in order to predict whether a given deoxyribonucleoside can be prepared by this enzymatic method a more systematic study was undertaken and the present report is a first attempt to define the structural requirements for a pyrimidine base to be a substrate for nucleoside deoxyribosyltransferase-II using thymidine as donor.

Materials and Methods

Reagents. The pyrimidine bases were commercial products from Sigma, Aldrich, Schwarz or Fluka. 3-Oxauracil was a gracious gift from Dr. J. Farkas (Prague) and 5-(3-phenylpropyl)-uracil was generously provided by Dr. B.R. Baker (Santa-Barbara). The purity was routinely checked by thin-layer chromatography and ultraviolet spectroscopy and when necessary the compounds were purified by paper chromatography as described in a previous report [14]. [^{14}C]Deoxyribosylthymidine was prepared as described earlier. For transfer studies the specific radioactivity was lowered to 2 Ci/mol. Phosphate buffers were prepared with sodium salts.

Enzyme. Nucleoside deoxyribosyltransferase was extracted from *L. helveti-*

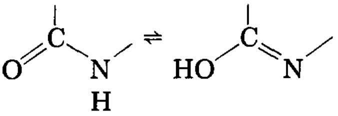
cus (NCDO 30, CNRZ collection, Jouy-en-Josas) and purified by affinity chromatography [4]. The fraction used contained about 3% of the (Pur ⇌ Pur) transfer activity using the (dIno ⇌ Ade) transfer test. This activity is close to what one would expect from a pure nucleoside deoxyribosyltransferase-II fraction. (Pur ⇌ Pyr) specific activity: 0.1 U/ml as defined in [15].

Transfer tests. The reagents were mixed in a 5 ml test tube in the order: [^{14}C]deoxyribosylthymidine, 0.1 μmol; pyrimidine base, 0.1 μmol (both substrates were dissolved in a 1 mM phosphate buffer, pH 6.0); enzymes, 0.1 ml; total volume, 0.5 ml. The reaction was performed at 40°C and two aliquots were taken after 1 h and 2 h. The samples were analysed essentially as described in a previous report [12] by thin-layer chromatography using six developing solvents. From the radioactivity profile recorded with an integrating radioactivity scanner (Chromelec 101, Numelec, Versailles, France) the extent of reaction was computed and occasionally the equilibrium constant was estimated when the reaction percentages after 1 h and 2 h were practically identical.

Results

Various substituents on the different positions of the pyrimidine ring were tried in order to obtain the best possible picture of the structural requirements for a pyrimidine base to be a competent acceptor.

Evidently the base must have a suitable NH function. This condition is fulfilled with a tautomeric equilibrium such as:



2-(1H)-Pyrimidinone is the simplest substituted pyrimidine complying with the necessary condition and was found to be a good substrate, as also observed by Votruba et al. [16].

Now the specificity of the enzyme will arise from interactions of the substituents with the active site of the enzyme or from the effect of these substituents on parts of the substrate interacting with the active site.

Position 2 and 4. Table I shows that when a 2-hydroxy substituent is present a variety of other substitutions such as OH (uracil), NH_2 (cytosine) and even CH_3 (4-methyl-2-pyrimidinol) are acceptable on position 4. These groups differ in nature, indicating that this part of the substrate has probably no close interaction with the enzyme. The nature of a good substituent on position 2 was defined by testing compounds with a suitable group on position 4. As expected the reaction was positive with 2-thio-4-pyrimidinol or 4-amino-2-pyrimidinethiol where SH replaced the OH group adjacent to N-1, whereas 2-amino-4-pyrimidinol (isocytosine) was not a substrate.

Position 5 (Table II). A substituent on this position was generally accepted, as can be seen from the variety of 5-substituted compounds which were found to be substrate. However, 5-(3-phenylpropyl)-uracil and 5-uracil carboxylic acid gave no apparent reaction and 5-nitrouracil reacted very slowly.

TABLE I

Pyrimidine bases with substituents on positions 2, 4 and 5 as substrates of nucleoside deoxyribosyltransferase-II. The transfer tests were performed with thymidine as the donor (see Materials and Methods). *K*, apparent equilibrium constant; in this column: + indicates a positive reaction, —, no significant reaction. Percent (2 h), percent of reaction after 2 h. This value is given instead of *K* when it was assessed that the reaction did not reach an equilibrium.

Substrate analogue	R2	R4	R5	*K*	% (2 h)
2-Pyrimidinol	-OH	—	—	+	35.0
Uracil	-OH	-OH	—	0.86	
Cytosine	-OH	$-NH_2$	—	1.93	
Thymine	-OH	-OH	CH_3	+	
2-Thio-4-pyrimidinal	-SH	-OH	—	+	16.3
4-Amino-2-pyrimidinethiol	-SH	$-NH_2$	—	0.05	
2-Thio-5-methyl-4-pyrimidinol	-SH	-OH	CH_3	+	10.8
2-Aminopyrimidine	$-NH_2$	—	—	—	
2-Amino-4-pyrimidinol	$-NH_2$	-OH	—	—	
4-Pyrimidinol	—	-OH		0.40	
4-Methyl-2-pyrimidinol	OH	$-CH_3$		+	

TABLE II

Pyrimidine bases with substituents on positions 2, 4 and 5 as substrates of nucleoside deoxyribosyltransferase-II. Conditions and symbols as indicated in Table I.

Substrate analogue	R2	R4	R5	*K*	% (2 h)
5-Hydroxyuracil	-OH	-OH	-OH	+	34.8
5-Fluorouracil	-OH	-OH	-F	0.84	
5-Bromouracil	-OH	-OH	-Br	0.79	
5-Hydroxymethyluracil	-OH	-OH	$-CH_2OH$	+	16.4
Thymine	-OH	-OH	$-CH_3$	+	
5-Uracilcarboxylic acid	-OH	-OH	-COOH	—	
5-(3-Phenylpropyl)-uracil	-OH	-OH	$-(CH_2)_3-\phi$	—	
5-Nitrouracil	-OH	-OH	$-NO_2$	+	7.5
5-Methylcytosine	-OH	$-NH_2$	$-CH_3$	+	34.1
5-Thiouracil	-OH	-OH	-SH	+	

TABLE III

Pyrimidine bases with substituents on positions 2, 4—6 as substrates of nucleoside deoxyribosyltransferase-II. Conditions and symbols as indicated in Table I.

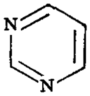
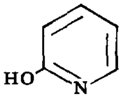
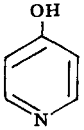
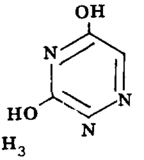
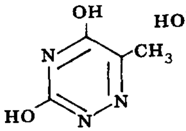
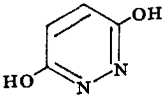
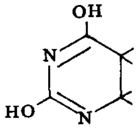
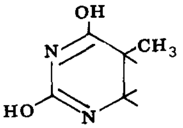
Substrate analogue	R2	R4	R5	R6	*K*	% (2 h)
Barbituric acid	-OH	-OH	—	-OH	—	
6-Methyluracil	-OH	-OH	—	CH_3	—	
5,6-Dimethyluracil	-OH	-OH	$-CH_3$	$-CH_3$	—	
6-Chlorothymine	-OH	-OH	—	-Cl	—	
4,6-Pyrimidinediol	—	-OH		-OH	+	45.0
6-Methyl-2-thio-4-pyrimidinol	-SH	-OH		$-CH_3$	—	
6-Phenyl-2-thio-4-pyrimidinol	-SH	-OH		$-\phi$	—	
6-Propyl-2-thio-4-pyrimidinol	-SH	-OH		$-C_3H_7$	—	
4-Amino-6-pyrimidinol		$-NH_2$		-OH	+	
2-Methyl-4,6-pyrimidinediol	$-CH_3$	-OH		OH	—	

Position 6. Adding a substituent on position 6 to bases which where otherwise good substrates always resulted in a negative transfer reaction. Table III shows that for a variety of substituents, either bulky such as propyl- or phenyl- or as small as methyl-, the reaction was negative. Again this reaction is negative when a polar group (barbituric acid) is present on this same position suggesting a simple steric hindrance effect as observed for position 8 of the purines [12]. However, 4,6-pyrimidinediol and 4-amino-6-pyrimidinol were good substrates whereas 2-methyl-4,6-pyrimidinediol did not react. These apparent exceptions will be discussed together with the case of 4-pyrimidinol.

Modification of the pyrimidine ring (Table IV). The available analogues showed that the replacement of carbon by a nitrogen on position 6 (*as*-triazine-3,5-(2H,4H)-dione (6-azauracil) and 6-methyl-*as*-triazine-3,5-(2H,4H)-dione (6-azathymine) gave a competent acceptor but the reaction was certainly slower than for the corresponding natural substrates uracil and thymine. 2-Pyridinol and 4-pyridinol failed to react, suggesting a role for N-3 whereas 3,6-

TABLE IV

Pyrimidine base analogues as substrates of nucleoside deoxyribosyltransferase-II. Conditions and symbols as indicated in Table I.

Substrate analogue		*K*	% (2 h)
Pyrimidine		—	
2-Pyridinol		—	
4-Pyridinol		—	
as-Triazine-3,5-diol		0.09	
6-Methyl-*as*-triazine-3,5-diol		+	4.2
3,6-Pyridazinediol		+	
5,6-Dihydrouracil		—	
5,6-Dihydrothymine		—	

pyridazinediol was a good substrate. On the other hand compounds such as 5,6-dihydrouracil and 5,6-dihydrothymine were not acceptors indicating the essential part played by the aromatic ring.

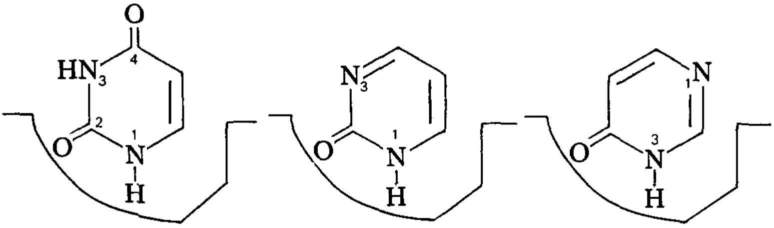
Discussion

The structural requirements for a pyrimidine base to be an acceptor may be summarized by a first approximate hypothesis:

(1) A lactim-lactam type tautomerism must be possible between the nitrogen atom N-1 and a suitable group on an adjacent atom of the ring. (2) No substituent can be present on the other adjacent atom for steric reasons. (3) The aromatic properties of the ring must be preserved.

In pyrimidines and related heterocyclic analogues there are various ways of providing the necessary -NH group. An adjacent -OH or -SH group is best suited since many ultraviolet, infrared and NMR studies [17] show the keto and thione forms to be favored in aqueous solution whereas in amino derivatives the imino form is negligible. However, this does not preclude the possibility that on the enzyme active site the substrate may assume some other tautomeric configuration. Since 4-pyrimidinol was shown to be a competent acceptor this observation raises the question of whether (a) the tautomeric group must be adjacent to the deoxyribosyl acceptor nitrogen atom in which case the keto-enol (or thione-thiol) group plays an essential part for binding and/or during catalysis, or (b) the N-3 nitrogen is essential. The case of 4-pyrimidinol indicates that these two possibilities are mutually exclusive. For 4-pyrimidinol in aqueous solution at 25—30°C Beak et al. [18] found $K_T > 4$ (K_T = N3H/OH) strongly suggesting that N-3 instead of N-1 is the deoxyribosyl-accepting site.

To help in the discussion, uracil will be taken as the standard substrate fitting 'normally' in the enzyme active site (I). The enzyme regions close to the various positions of the substrate ring will be designated according to the corresponding ring atom and substituent numbers. Thus the position of the substrate on the enzyme is governed by the iminol-amide group fitting in the 1—2 enzyme region such as in:



I II III

Scheme I

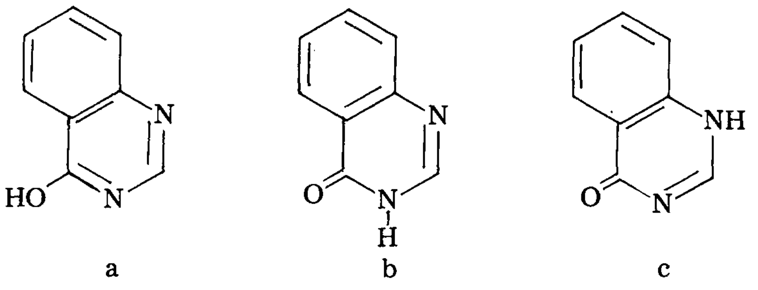
and the role of the second nitrogen (N-3 in uracil) seems to be of minor importance.

This is corroborated by the following observations:

(1) 3-Methyluracil gave a positive reaction, indicating that N-3 does not interact directly with the enzyme.

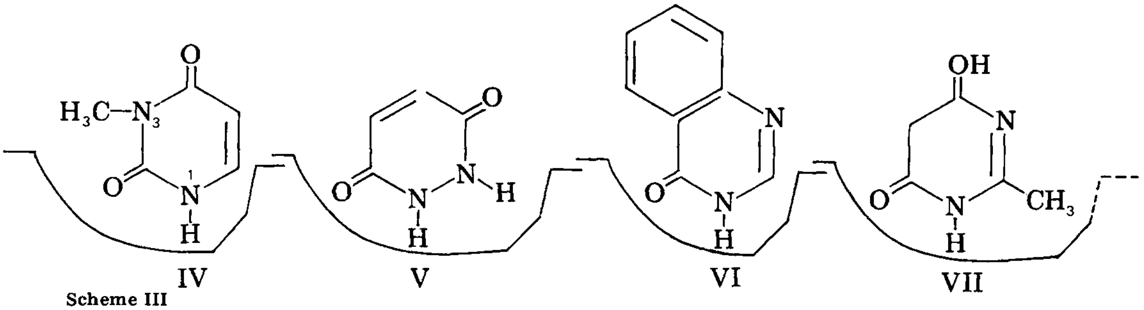
(2) 3,6-Pyridazinediol may be considered as a symmetrical double amide and shows that the place of the second nitrogen in the ring is not essential as this compound was a good acceptor.

(3) 4-Quinazolinol might have any of the three structures:



Scheme II

According to Hearn et al. [19] comparing ultraviolet spectra with those of the corresponding methyl derivatives, the relative proportions of each species were, respectively, a: 20%; b: 70%; c: 10%. Again this compound was a substrate and it is assumed to position on the enzyme as in VI showing that a bulky group in the '3—4' region of the enzyme is well tolerated.



Scheme III

On the other hand tests performed with 2-pyridinol and 2H-1,3-oxazine-2,6-(3H)-dione (oxauracil) were negative so the second nitrogen might serve to ensure an adequate electronic structure of the ring.

Point 2 of the hypothesis is sustained by the fact that thymine or uracil derivatives with a substituent on the 6 position were all found negative whether this substituent was polar (barbituric acid) or hydrophobic (6-methyluracil or 5,6-dimethyluracil).

4,6-Pyrimidinediol is a substrate because again the 1—6 position can fit in the enzyme 1—2 region whereas 2-methyl-4,6-pyrimidinediol does not react because when the 1—6 position occupies the enzyme 1—2 region the methyl group is in the hindered 6 region (VII). An analogous situation is found with 2-amino-4-(3H)-pyrimidinone (isocytosine). Indeed this tautomeric form represents only 48% of the isocytosine present in aqueous solution, the other form being 2-amino-4-(1H)-pyrimidinone [20]. The fact that the tests were negative provides interesting confirmation that the deoxyribosyl-accepting nitrogen is necessarily adjacent to the tautomeric OH or SH. Scheme III suggests that 4-pyrimidinol is substituted at N-3.

An exception to point 2 was found with xanthine which formed some 3-(2-

deoxy-β-D-ribofuranosyl) derivatives, as well as the main reaction products i.e. 7- and 9-(2-deoxy-β-D-ribofuranosyl)-xanthine, when incubated with thymine in the presence of the enzyme.

Position 3 of xanthine appears to have an enhanced reactivity moreover a comparison of the N-3—O-4—N-9 angle in xanthine [21] with the corresponding N-1—C-6—C′-6 angle of 5-ethyl-6-methyluracil [22] and N-1—C-6—O′-6 of barbituric acid [23] shows very significant differences (respectively, 13.5° and 4.5°) which may explain why the steric hindrance expected from N-9 of xanthine nevertheless permits a non-negligible binding of this substrate. This point is an extension of a previous study [24] and will be discussed in detail in a separate report.

The third point of the hypothesis is based mainly on the observation that 5,6-dihydro derivatives were not substrates suggesting that the delocalized electrons play an essential role in binding.

In relation to a previous study on the specificity of this same enzyme for purine bases [12] we observe that the same type of structural requirement is present in both cases, i.e. a structure such as a tautomeric shift between the reacting nitrogen and another correctly situated group is preserved. In pyrimidine as well as in purine an adjacent steric hindrance is also critical (cf. the 8 position of the purines). It was shown that the transfer of the deoxyribosyl group catalyzed by nucleoside deoxyribosyltransferase-II proceeds via a ping-pong-bi-bi mechanism [15], implying that from the same deoxyribosyl-enzyme complex the deoxyribosyl group is transferred either to a pyrimidine or a purine base. The question arises as to whether the two kinds of acceptor substrates (purines and pyrimidines) bind onto the same point or whether there are two distinct subsites for the substrates.

Acknowledgements

We wish to express our gratitude toward the late Dr. B.R. Baker for the gift of 5-(2-phenylpropyl)-uracil and Dr. J. Farkas for a sample of 3-oxauracil. This work was supported in part by a predoctoral fellowship from the Commissariat à l'Energie Atomique to J.H.

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