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Note

Dissociation constants and dissociation rate of the nojirimycin–hydrogen sulfite adduct and related compounds

GÜNTER LEGLER AND WILMA BECHER
Institut für Biochemie der Universität Köln, Zülpicher Str. 47, D-5000 Köln 1 (Germany)
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The antibiotic nojirimycin (5-amino-5-deoxy-D-glucose) is normally isolated and stored as its hydrogen sulfite addition-compound[1]. Nojirimycin is a strong inhibitor of glycosidases[2,3], with a slow approach to the equilibrium of the enzyme–inhibitor complex[4,5]. In some cases[3,4], the measurements were made with the hydrogen sulfite adduct, with the tacit assumption of complete dissociation. An anomalous inhibition-pattern was observed with a β-D-glucosidase from almonds, which showed[4] an apparent negative co-operativity with a Hill coefficient (n_H) of 0.51. We have confirmed this finding and noticed, in addition, a biphasic approach to the steady-state rate[6]. Both anomalies were observed only with the hydrogen sulfite adduct, and not with free nojirimycin where no co-operativity could be detected and where the approach to the steady state of substrate hydrolysis could be described by a single rate-constant. In order to clarify this situation, we have determined the dissociation constant of the adduct and its dissociation rate by a simple photometric method based on the rapid oxidation of hydrogen sulfite by the iodine–starch complex. Two aliphatic 1-amino-1-sulfonic acids were studied for comparison.

When a sufficiently dilute solution of the nojirimycin–hydrogen sulfite adduct was added to iodine–starch, an immediate fall of the absorbance A_{570} was observed, followed by a slow, exponential decrease (Fig. 1). Control experiments showed that free hydrogen sulfite added in substoichiometric amounts to the iodine–starch solution caused a rapid decrease in A_{570} that reached its final value in <5 s. It was also shown that free nojirimycin and the other aldehydes studied were oxidized by the iodine–starch complex at rates at least two orders of magnitude lower than the absorbance decrease following the initial, rapid drop of A_{570} (1.8×10^{-3} min^{-1} for nojirimycin; 0.7×10^{-6} and 150×10^{-6} min^{-1} for formaldehyde and acetaldehyde, respectively, with 20 μM iodine–starch at pH 6.0). Therefore, it may be assumed that the initial fall in A_{570} is due to free hydrogen sulfite in equilibrium with the adduct before its addition to the iodine solution and that the slow decrease is due to its dissociation in the cuvette. Our results for the equilibrium constants calculated from the initial

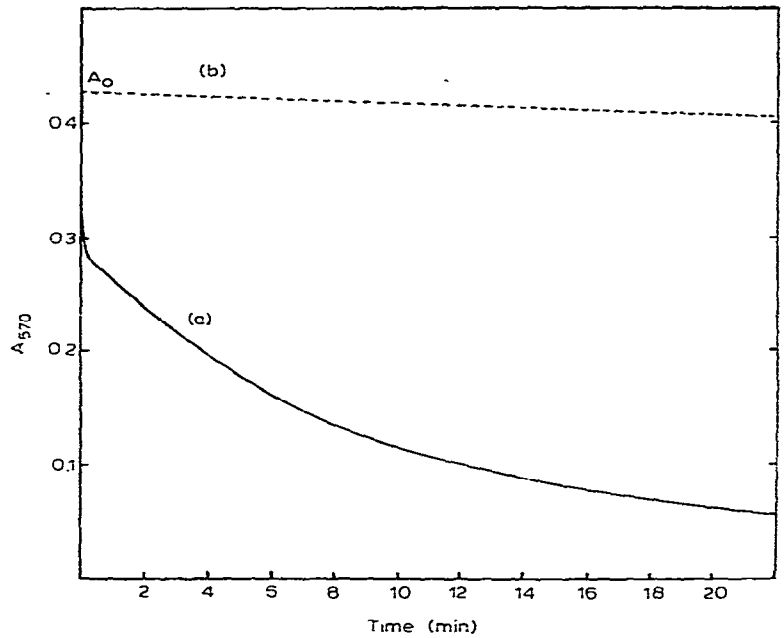


Fig. 1. Time course of the absorbance A_{570} of 20 μM iodine–starch (final concentration) on addition of 0.5 ml of 60 μM nojirimycin–hydrogen sulfite (a) or 60 μM free nojirimycin (b) to a final volume of 2.1 ml; A_0, absorbance reached on addition of 0.5 ml of buffer. Conditions: pH 4.0 and 25°.

fall in A_{570} and the dissociation rates of the nojirimycin adduct and of the corresponding derivatives of formaldehyde and acetaldehyde are summarized in Table I.

The data show that the nojirimycin adduct is only partially dissociated at the concentrations used for the inhibition studies in refs. 3 and 4, and that the apparent negative co-operativity[4], with $n_H = 0.51$, can be explained by the square-root dependence of the concentration of free nojirimycin in solutions of the adduct when [adduct] $\gg K_{dis}$. It also follows from $n_H = 0.51$ that any inhibition by the adduct can be neglected in comparison to that caused by free nojirimycin.

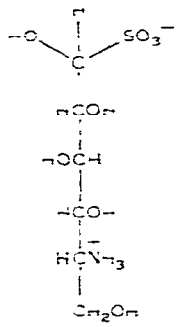
The comparable magnitude of the dissociation constants of the adducts from

TABLE I

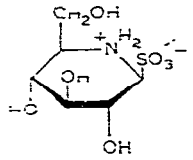
DISSOCIATION CONSTANTS AND DISSOCIATION RATES OF 1-AMINO 1-SULFONIC ACIDS DERIVED FROM NOJIRIMYCIN, FORMALDEHYDE, AND ACETALDEHYDE AT 25°

Aldehyde	*pH*	K_{dis} *(μM)*	k_{dis} *(min^{-1})*
Nojirimycin	4.0	15.5 ±0.8	0.17 ±0.01
Nojirimycin	5.0	3.2 ±0.2	0.20 ±0.01
Nojirimycin	6.0	1.5 ±0.1	0.27 ±0.015
Formaldehyde	6.0	0.07 ±0.01	0.007 ±0.001
Acetaldehyde	6.0	3.7 ±0.1	0.22 ±0.01

nojirimycin and the aliphatic aldehydes raises the question of the structure of the former. While Inouye *et al.*[1] gave the acyclic structure **1**, ^{1}H-n.m.r. data[7] favour the cyclic structure **2**.



1



2

Published data on the dissociation constants of aldehyde–hydrogen sulfite adducts[8] are scarce and do not permit an unequivocal distinction between the two isomers. We have therefore determined the proton dissociation constant of the nojirimycin adduct, because that of **1** should be similar to that of 2-amino-2-deoxy-D-glucose (pK_a 7.71) while that of **2** should be ~5 pK_a units lower, as seen from a comparison of the values for methylamine (pK_a 10.66) and aminomethanesulfonic acid[10] (pK_a 5.75). Potentiometric titration of the hydrogen sulfite adduct gave pK_a 3.50 ±0.03, thus supporting the cyclic structure **2**.

EXPERIMENTAL

The nojirimycin–hydrogen sulfite adduct was a gift from Dr. S. Inouye, Yokohama. A solution of the adduct (200 mg) in water (15 ml) was stirred with Dowex 1-X8 (HO^-) resin (5 g) for 1 h and then filtered over charcoal, and the free base was recovered by lyophilisation. Part of the material was used to determine its oxidation rate, and the rest was reconverted[1] into the hydrogen sulfite adduct and dried *in vacuo* over solid NaOH for 24 h. Aminomethanesulfonic acid was obtained from Fluka, and 1-aminoethane-1-sulfonic acid was prepared according to the method of Backer and Mulder[11] and crystallized once from water. Contamination by free hydrogen sulfite was <0.3%.

Oxidation and dissociation rates were measured with a Zeiss PMQ 2 spectrophotometer (equipped with a Servogor S recorder and 1-cm cuvettes thermostatted at 25 ±0.2°) by injecting appropriately diluted solutions of the hydrogen sulfite adduct or free aldehyde into buffered solutions of the iodine–starch complex. Buffers were 0.1M sodium acetate–HCl (for pH 4.0 and 5.0) and 0.05M Na_2HPO_4–HCl (for pH 6.0). The final concentration of iodine was 20μM, and that of soluble starch (Merck, Darmstadt) was 0.6 g/L. Rates were calculated from the slopes of recorder traces of A_{570} *vs.* time, with ε_{570} for the iodine–starch complex. Since slight variations

in the preparation and age of the starch solution caused ε_{570} to vary between 21.5 and 24.3 $mM^{-1}cm^{-1}$, it was re-determined for each set of measurements.

The degree of dissociation (α) was calculated from the initial, rapid drop of A_{570} and the concentrations (c_0) of the stock solutions of hydrogen sulfite adducts (0.5–0.01 mM) that had been left to equilibrate for at least 1 h at 25°. A standard curve of A_{570} *vs.* added sodium hydrogen sulfite was linear down to $A_{570} \sim 0.02$ where dissociation of the iodine–starch complex became noticeable, causing a downward curvature. Dissociation constants K_{dis} were calculated with $K_{dis} = [\alpha^2/(1 - \alpha)]c_0$ for five different concentrations (c_0) of the nojirimycin and acetaldehyde derivatives so that α was between 0.05 and 0.5. Measurements with aminomethanesulfonic acid could only be carried out at concentrations where α ranged from 0.01 to 0.04.

The proton dissociation constant of the nojirimycin–hydrogen sulfite adduct was determined by potentiometric titration of a 0.02M solution with 0.1M NaOH. Back titration with 0.1M HCl gave the same pH at half-neutralisation (within 0.05 unit), indicating that practically no decomposition had taken place.

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