# THE USE OF PARAMAGNETIC $^{13}C$ NMR RELAXATION TO STUDY THE MECHANISMS OF THE AMINO ACID ACTIVATION CATALYSED BY A COGNATE tRNA SYNTHETASE

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## 1. Introduction

The activation reaction:

$$AA + ATP + E \rightleftarrows X \rightleftarrows E \cdot AA - AMP + PP_i \qquad (1)$$

by which a specific tRNA synthetase (E) catalyses the adenylation of its cognate amino acid (AA), before it is transferred to the corresponding tRNA, requires the presence of divalent ions, usually $Mg^{2+}$. Other divalent ions, such as $Mn^{2+}$, $Ni^{2+}$ or $Co^{2+}$, can substitute for $Mg^{2+}$ [1,2].

Detailed studies of the mechanisms of reaction (1), including the role of the enzyme intermediate, X, have been made for specific enzyme systems, in the presence of $Mg^{2+}$ [3,4] and $Mn^{2+}$ [5] using the conventional ATP–$PP_i$ exchange method, or fluorescence stopped-flow technique.

Here, we report the results of a study of reaction (1) for the glycine, *Escherichia coli* glycyl-tRNA synthetase (gtRS), ATP-system in the presence of $Mn^{2+}$, obtained from $^{13}C$ NMR relaxation measurements of the glycine substrate. As will be demonstrated, this approach, in combination with the paramagnetic property of $Mn^{2+}$, presents a very sensitive and informative method to probe the dynamics and structures of the intermediate substrate–enzyme complexes in the reaction.

## 2. Materials and methods

The 4-subunit ($\alpha_2\beta_2$) gtRS was isolated from *E. coli* cells, strain SØ929, according to [6]. Protein was determined by the method in [7]. A determination of $M_r$ using SDS–polyacrylamide gel electrophoresis [8] gave $M_{r,\alpha}$ 40 000 and $M_{r,\beta}$ 80 000. The specific activity of the freshly purified enzyme was 24 000 units/mg, where one unit is defined as the formation of 1 nmol $^{32}PP_i$ from [$^{32}$P]ATP in 10 min at 37°C [6]. To stabilize the enzyme and extend the experimental temperature range, a mixture of 60/40 vol% $H_2O$/glycerol was used as a solvent for the NMR samples, which, furthermore, were kept in NMR tubes, sealed in vacuo. Whenever not in use the samples were stored at –20°C. Besides phosphate (20 mM) and β-mercaptoethanol (5 mM), the enzyme-free sample (sample I) contained ATP (0.125 mM), glycine (0.125 M, 90% [$^{13}$C(1)], 0.125 M, 90% [$^{13}$C(2)]), and $Mn^{2+}$ (0.075 mM), while the enzyme-containing sample (sample II) was identical to sample I, except for the presence of gtRS (4 mg/ml). The measured pH was 7.4 in all cases.

The $^{13}C$ NMR measurements were accomplished at 67.8 MHz and 22.6 MHz using Bruker HX 270 and WH 90 spectrometers. The $T_1$-values were obtained with the $T_{Rep}-180°-\tau-90°$ pulse sequence. A non-linear, 3-parameter least-squares analysis of the experimental line intensities [9] resulted in $T_1$-values with 1σ standard deviations <2%, while the linewidths yielded $T_2$ data with an estimated uncertainty of 5%. The paramagnetic contributions were determined as $T_{kp}^{-1} = T_{k,obs}^{-1} - T_{kA}^{-1}$ ($k = 1,2$), where $T_{k,obs}^{-1}$ is the rate in $Mn^{2+}$-containing samples, and $T_{kA}^{-1}$ is the corresponding rate in a solution without $Mn^{2+}$ (sample III), but otherwise identical to sample I.

The $T_{kp}$ data were analyzed using a general 3-site exchange model [10], which relates these data to the exchange rates, $\tau_M^{-1}$, and the relaxation rates $T_{1i}^{-1}$, $T_{2j}^{-1}$ of the $Mn^{2+}$-bound glycine. For the simpler 2-site exchange scheme this model reduces to the well-known [11], Luz-Meiboom [12] and Swift-Connick equations [13]. $T_{1j}^{-1}$ and $T_{2j}^{-1}$ are, in turn, given by the modified Salomon-Bloembergen equations [11,14,15]:

$$\frac{1}{T_{1j}} = \frac{2}{15}\frac{S(S+1)g^2\beta^2\gamma_I^2}{r_j^6}\left[\frac{3\tau_{c,1}}{1+\omega_I^2\tau_{c,1}^2} + \frac{7\tau_{c,2}}{1+\omega_S^2\tau_{c,2}^2}\right] + \frac{2}{3}S(S+1)\left(\frac{A}{\hbar}\right)^2\left[\frac{\tau_{e,2}}{1+\omega_S^2\tau_{e,2}^2}\right] \quad (2)$$

$$\frac{1}{T_{2j}} = \frac{1}{15}\frac{S(S+1)g^2\beta^2\gamma_I^2}{r_j^6}\left[4\tau_{c,1} + \frac{3\tau_{c,1}}{1+\omega_I^2\tau_{c,1}^2} + \frac{13\tau_{c,2}}{1+\omega_S^2\tau_{c,2}^2}\right] + \frac{1}{3}S(S+1)\left(\frac{A}{\hbar}\right)^2 \times \left[\tau_{e,1} + \frac{\tau_{e,2}}{1+\omega_S^2\tau_{e,2}^2}\right] \quad (3)$$

where, $\tau_{c,k}^{-1} = \tau_R^{-1} + T_{ke}^{-1} + \tau_M^{-1}$ and $\tau_{e,k}^{-1} = T_{ke}^{-1} + \tau_M^{-1}$, $\tau_R^{-1}$ and $T_{ke}^{-1}$ are the isotropic reorientation rates of the $Mn^{2+}$-bound glycine and the relaxation rates of the unpaired electrons, respectively. For the present system the relation, $T_{2e}^{-1} \simeq T_{1e}^{-1}$, holds to a good approximation, leaving $\tau_{c,1}^{-1} = \tau_{c,2}^{-1} = \tau_c^{-1}$ and $\tau_{e,1}^{-1} = \tau_{e,2}^{-1} = \tau_e^{-1}$, while:

$$\frac{1}{T_{1e}} = \frac{\Delta^2}{25}\left[4S(S+1) - 3\right]\left[\frac{\tau_v}{1+\omega_S^2\tau_v^2} \pm \frac{4\tau_v}{1+4\omega_S^2\tau_v^2}\right] \quad (4)$$

Here, $\tau_v$ is the corresponding correlation time and $\Delta$ is the zero field splitting parameter.

### 3. Results and discussion

Primarily, the substantial $T_{kp}^{-1}$ rates found for the glycine carbons in both $Mn^{2+}$-containing samples (fig.1) show, qualitatively, that glycine forms first coordination sphere complexes with $Mn^{2+}$. No paramagnetic effect on the solvent glycerol carbons were observed. Furthermore, in sample II any non-paramagnetic contributions to the $T_{kp}^{-1}$ rates caused by possible associations between glycine and gtRS without the involvement of $Mn^{2+}$, are negligible. Thus, the dramatic increases of $T_{kp}^{-1}$, caused by 0.017 mM gtRS in sample II at the lowest temperatures, as compared to sample I, are similar in magnitude to what is caused by a comparable concentration of $Mn^{2+}$ in sample I.

Even with the most favorable correlation time, $\tau_c = \omega_I^{-1}$, for the relaxation of the glycine carbons of a pure glycine–gtRS associate, a measurable contribution from this species would necessitate unrealisticly large values of the prefactors in eq. (2) and (3) should the increased $^{13}C$ relaxations be caused by other mechanisms than interactions with unpaired electrons. Additionally, one would expect such a contribution to be larger for the proton-bearing C(2) than for the quaternary C(1), while experimentally the effect is substantially larger for C(1) than C(2). Hence, the data obtained from sample I and II unambiguously show, that the gtRS-induced increases of the observed relaxation rates are due to an interaction between gtRS and glycine, which involves $Mn^{2+}$.

Secondly, the disappearance at high temperatures of the gtRS-induced increases of $T_{kp}^{-1}$ shows, that they are mainly due to changes in the correlation rates, rather than to an increase of the number of paramagnetic sites available for the glycine, which could have been caused by a draining of the competing ligand, ATP, away from the bulk solution, through the formation of a $Mn^{2+}$-free gtRS · ATP complex. This interpretation is conclusively supported by the considerably smaller $T_{1p}^{-1}$ rates [16], which are observed for the glycine carbons in an ATP-free solution which is otherwise identical to sample I. The slopes of the temperature dependences of the two types of relaxation rates show, furthermore, that the gtRS-induced change, in the case of $T_{1p}^{-1}$, is mainly due to a change in the correlation rate controlling $T_{1j}^{-1}$, while it, in the $T_{2p}^{-1}$ case, is caused by an increase of $\tau_M^{-1}$. Thus the data in fig.1a,b qualitatively show, that gtRS decreases $\tau_{c,1}$ for the glycine carbons and increases their exchange rate, $\tau_M^{-1}$, from the $Mn^{2+}$-bound positions.

Thirdly, the dramatic increase of all $T_{1p}^{-1}$ rates with decreasing field strength, observed at low temperatures, shows that $\omega^2\tau^2 \gg 1$ is the dominating term in eq. (2), which allows a determination of $\tau$. The field dependence of $T_{2p}^{-1}$, which is most pronounced at high temperature due to a decreasing influence from $\tau_M^{-1}$, is the opposite of what is observed for $T_{1p}^{-1}$. The latter shows not only that $T_{1j}^{-1}$ and $T_{2j}^{-1}$ are controlled by different correlation rates, but also that it, in the case of $T_{2j}^{-1}$, itself is field dependent and therefore must be influenced by the only field-dependent correlation rate, $T_{1e}^{-1}$.

Quantitatively, a non-linear, least squares analysis of the data in fig.1 shows that the data from sample I conform to a simple two-site exchange process,

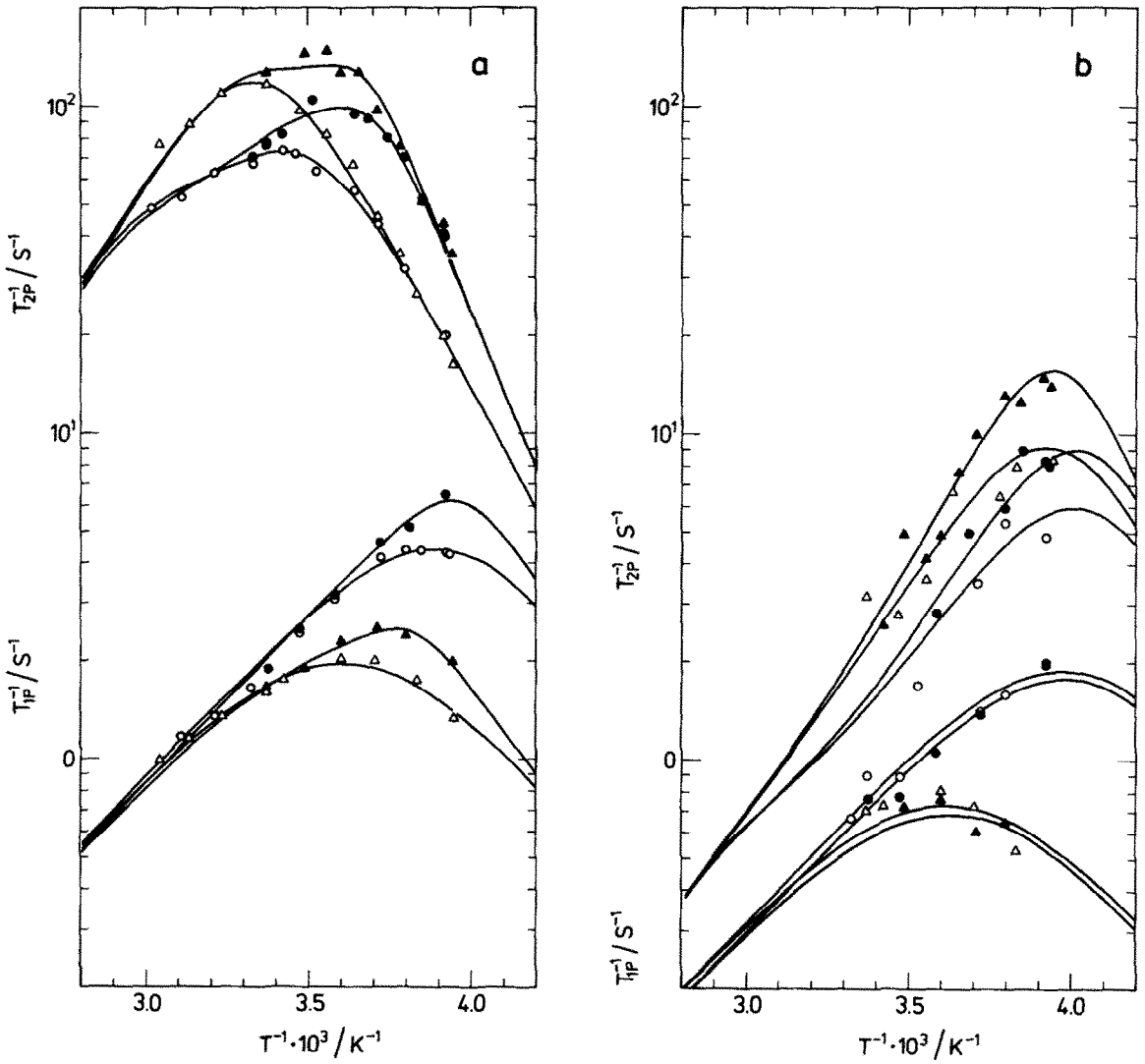


Fig.1. Temperature variation of the paramagnetic contributions to the relaxation rates of the glycine carbons (a) C(1) and (b) C(2) obtained at 22.6 MHz (○,●) and 67.9 MHz (△,▲). As to further specification of the samples see section 2. The upper two curves are the $T_{2p}^{-1}$ rates, the lower two curves the $T_{1p}^{-1}$ rates. (○,△) Refer to the gtRS-free sample I; (●,▲) to the gtRS-containing sample II. The curves were calculated using the parameters in table 1.

$A \rightleftarrows B$, where A is the bulk glycine and B the $Mn^{2+}$-bound glycine. Furthermore, the analysis shows, that $T_{1j}^{-1}$ is entirely controlled by the dipolar term, while only the scalar term contributes to $T_{2j}^{-1}$. Although the obtained absolute value of $\tau_R$ is ~50% longer than what is found for the ATP-free solution [16], and therefore indicates that an ATP · $Mn^{2+}$ · glycine complex is present, it may not entirely exclude a pure glycine · $Mn^{2+}$ complex. The remaining of the model parameters depend on the ratio, $P_M$, between the $Mn^{2+}$-bound and the unbound glycine, which cannot be obtained from the experimental data set. In order to eliminate this indeterminate character of $P_M$, a value of 3.00 Å was assumed for the C(1)–$Mn^{2+}$ distances, on the basis of other divalent metal complexes of amino acids [17]. The parameter values thus obtained are given in table 1. A calculation with $P_M$ = [$Mn^{2+}$]/[glycine] gives a similar good fit but yields unrealistically slow $\tau_M^{-1}$ and $\tau_{1e}^{-1}$ rates combined with large $Mn^{2+}$–C distances and electron–carbon hyper-

Table 1
Parameters obtained from the least squares analysis of the experimental relaxation data

| Parameters | (units) | Complex B | Complex C |
|---|---|---|---|
| $\Delta H^{\ddagger}$ | (kcal/mol) | 7.95 ± 0.18 | 14.4 ± 0.3 |
| $\Delta S^{\ddagger}$ | (e.u.) | 0.0 ± 0.7 | 27.3 ± 0.9 |
| $\tau_M^{-1}$(298 K) × $10^{-7}$ ($s^{-1}$) | | (0.93 ± 0.03) | (15.1 ± 0.4) |
| $E_R$ | (kcal/mol) | 4.77 ± 0.06 | 13.28 ± 0.12 |
| $\tau_R^{-1}$(298 K) × $10^{-9}$ ($s^{-1}$) | | 0.802 ± 0.008 | 9.5 ± 1.2 |
| $E_v$ | (kcal/mol) | 8.5 ± 1.3 | 7.3 ± 6 |
| $\tau_v$ (298 K) × $10^{-11}$ ($s^{-1}$) | | 2.8 ± 0.8 | 2.3 ± 1.2 |
| $\Delta$ | (G) | 135 ± 9 | 210 ± 70 |
| $T_{1e}^{-1}$(298 K, 21.1 kG) × $10^{-7}$($s^{-1}$) | | 2.2 ± 0.7 | 4.8 ± 3 |
| $r(1)$ | (Å) | 3.00[a] | 3.00[a] |
| $r(2)$ | (Å) | 3.56 ± 0.02 | [b] |
| $A/\hbar$ (1) × $10^{-6}$ (Hz) | | 1.09 ± 0.02 | 1.56 ± 0.12 |
| $A/\hbar$ (2) × $10^{-5}$ (Hz) | | 0.83 ± 0.17 | 1.6 ± 0.8 |

[a] Assumed value (see text). The resulting value for $P_M$ was (4.6 ± 0.1) × $10^{-5}$ and (3.1 ± 0.1) × $10^{-5}$ for sample I and II, respectively
[b] Too large to affect the experimental data

fine coupling constants *(A)*, which, although small, are unrealistically large considering the $Mn^{2+}$–C distances.

The data obtained from sample II agree with a B ⇄ A ⇄ C exchange scheme, where C is the glycine bound in the $Mn^{2+}$ complex, that interacts with gtRS. A and B are as defined above. The parameter values for the C complex (table 1) were obtained assuming a set of B parameter values as found for the B complex in sample I (table 1), and a C(1)–$Mn^{2+}$ distance of 3.00 Å. However, the C parameters remained unchanged within the uncertainties, when assuming the condition $P_M = [Mn^{2+}]/[\text{glycine}]$ and applying the corresponding B parameter values. As for the B complex, $T_{1j}^{-1}$ and $T_{2j}^{-1}$ for the C complex are pure dipolar and scalar relaxations, respectively. The relative significance of the pertinent correlation rates for the two systems is illustrated in fig.2.

As it appears from table 1 and fig.2, the most conspicuous impact of gtRS on the $Mn^{2+}$-bound glycine is the dramatic increase of its mobility, both in terms of its dissociation rate, $\tau_M^{-1}$, and in terms of its mobility, $\tau_R^{-1}$, when bound to $Mn^{2+}$. In addition, gtRS causes a change in the position of the glycine molecule relative to $Mn^{2+}$, as judged from the increase of the relative C(2)–$Mn^{2+}$ distance.

According to the ATP–$PP_i$ exchange assay, the dissociation of the enzyme-bound adenylate following the reverse eq. (1) has a rate, which is orders of magnitude smaller than the obtained $\tau_M^{-1}$ value. Therefore, the highly unstable C complex must be identified as the transition intermediate, X, of eq. (1). In agreement with this identification it has been suggested [5], that the metal ion acts as a stabilizer of X. Thus the exchange parameters in table 1 characterise step 1 in eq. (1), while step 2 undoubtedly is too slow to affect the experimental data. The observation, that the $T_{kp}^{-1}$ rates remained unchanged during the period of the experiments, despite a reduction of the enzyme activity, as measured by the ATP–$PP_i$ exchange assay, by ~30% during the same period, strongly indicates that this reduction is due to a degradation of the enzyme that only affects step 2 in eq. (1). This indicates, combined with the mere fact that step 1 can occur, even though the active site is effectively occupied by the slowly converting adenylated glycine, that X is attached to a location in the enzyme, which is somewhat different from the site of the adenylate. The high dissociation rate of X could thus be stimulated by a failure of X to interact with this site in order to proceed according to step 2 in eq. (1).

The increased mobility, $\tau_R^{-1}$, of the C complex, may also be caused by this antagonistic effect, combined with a detachment of the $Mn^{2+}$-bound glycine from hydrogen bonding to the solvent glycerol, which undoubtedly occurs in the bulk solution. Although $\tau_R^{-1}$ (298 K) for the C complex is ~12-times faster than for the B complex, it is still ~3-times slower than for a similar glycine · $Mn^{2+}$ complex in pure solution [18].

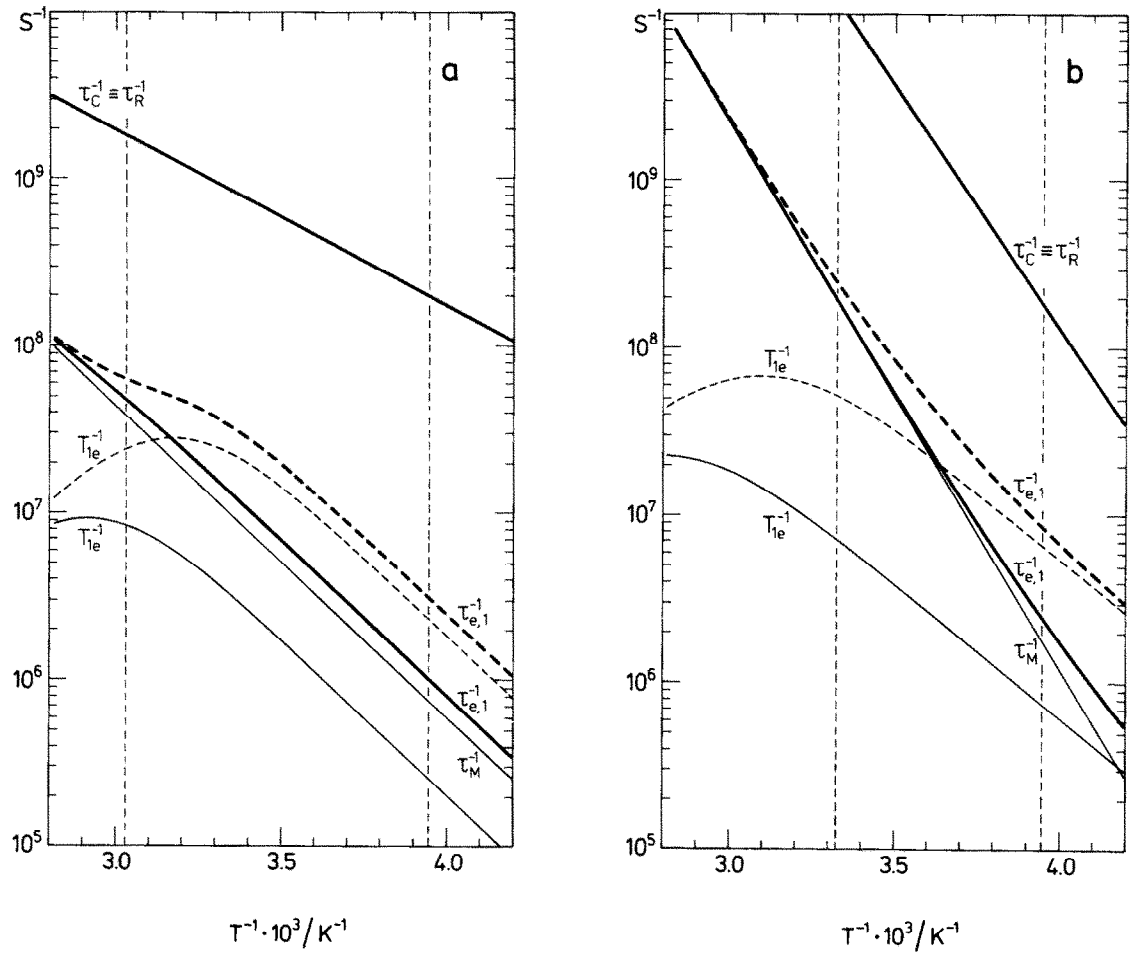


Fig.2. Temperature variation of the exchange rates, $\tau_M^{-1}$ [$= kT/h \times \exp(-\Delta H^{\neq}/RT + \Delta S^{\neq}/S)$] and the rotational reorientation rates, $\tau_R^{-1}$ [$= \tau_R^0 \times \exp(E_R/RT)$] of the ligand glycine in (a) the B complex and (b) the C complex. The $T_{1e}^{-1}$ curves denote the corresponding electron relaxation rates, the temperature variations of which are given by $\tau_v$ [$= \tau_v^0 \times \exp(E_v/RT)$] according to eq. (4). The appropriate sums, $\tau_c^{-1}$ and $\tau_e^{-1}$, are the effective correlation rates for $T_{1j}^{-1}$ and $T_{2j}^{-1}$, respectively (see text). The dashed, vertical lines indicate the experimental temperature regions.

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