FEBS Letters 390 (1996) 226–228 FEBS 17271

Probing the higher order structure of RNA with peroxonitrous acid

Matthias Götte[a], Roland Marquet[b], Catherine Isel[b], Vernon E. Anderson[c], Gérard Keith[b], Hans J. Gross[d], Chantal Ehresmann[b], Bernard Ehresmann[b], Hermann Heumann[a,*]

[a] *Max-Planck-Institut für Biochemie, Am Klopferspitz 18a, D-82152 Martinsried, Germany*
[b] *UPR no 9002 du CNRS, Institut de Biologie Moléculaire et Cellulaire, 15, rue R. Descartes, F-67084 Strasbourg Cedex, France*
[c] *Department of Biochemistry, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106, USA*
[d] *Bayerische Julius-Maximilians-Universität, Institut für Biochemie, Biozentrum, Am Hubland, D-97074 Würzburg, Germany*

Received 4 June 1996

Abstract Potassium peroxonitrite (ONOOK) and [Fe(EDTA)]$^{2-}$ were used to analyze the influence of chemically entirely different hydroxyl radical sources on tRNA cleavage profiles. [Fe(EDTA)]$^{2-}$ gives rise to hydroxyl radicals via a Fenton-like reaction during the oxidation of chelated Fe^{2+}, while ONOOK generates hydroxyl radicals via its conjugate acid (ONOOH) when adding a stable alkaline solution of ONOOK in samples buffered at neutral pH. [Fe(EDTA)]$^{2-}$ is known to induce oxidative strand scission at sugar moieties thought to be solvent accessible, while those residues located in the 'inside' of structured RNAs are protected. Although ONOOH is neutral and significantly smaller than the metal complex, both reagents generate the same protection pattern on tRNAs, suggesting that access of the commonly formed hydroxyl radical, rather than access of its source, is the determining factor when probing the higher order structure of RNA. Strong difference in reactivity is only seen at the modified 2-thiouridine S34 of tRNA^{Lys3} which shows hyperreactivity towards ONOOK treatment. This particular reaction may require interaction between the peroxonitrite anion and the thiocarbonyl group of the base, since hyperreactivity is not observed when probing the dethiolated tRNA^{Lys3}.

Key words: Chemical probing; Hydroxyl radicals; Fe-EDTA; ONOOK; tRNA

1. Introduction

Probing DNA or RNA with [Fe(EDTA)]$^{2-}$ promoted oxidation is a widely used technique to obtain information about nucleic acid structure or their interaction with proteins. Diffusible hydroxyl radicals generated by the modified Fenton reaction (Fig. 1, scheme 1) are thought to be the active species responsible for cutting the nucleic acid backbone ([1] and references therein). The hydroxyl radical presumably abstracts a hydrogen atom from a ribose carbon at C1′ and C4′, resulting in strand scission. [Fe(EDTA)]$^{2-}$ induces cleavage in single- and double-stranded regions with similar intensities, while reduced reactivity is observed when the sugar-phosphate backbone is in contact with another molecule or in those cases where the tertiary structure of the nucleic acid shields the sugar moiety. Latham and Cech [2] showed that treatment of tRNA^{Phe} with [Fe(EDTA)]$^{2-}$ yields cleavage only at solvent-accessible sugar moieties, while residues located in the 'inside' of correctly folded RNA are protected. It remained to be elucidated whether the observed protection pattern could be attributed to the inaccessibility of short-lived hydroxyl radicals or to the inaccessibility of the bulky metal-complex itself, that would prevent sufficient production of hydroxyl radicals in proximity to protected residues. We addressed this issue, analyzing the influence of chemically distinct hydroxyl radical sources on tRNA cleavage profiles. tRNA^{Phe} and tRNA^{Lys3} were probed with [Fe(EDTA)]$^{2-}$ and potassium peroxonitrite (ONOOK), which generates hydroxyl radicals via transiently formed peroxonitrous acid (ONOOH) in the absence of heavy-metals [3,4]. Generation of hydroxyl radicals is initiated immediately after protonation of the peroxonitrite anion ($ONOO^-$) when adding a stable alkaline solution of ONOOK into the sample solution, which is buffered at neutral pH (Fig. 1, scheme 2). This method has successfully been applied to footprint DNA-protein complexes [4].

2. Materials and methods

Rabbit tRNA^{Lys3} with the anticodon SUU (S = mcm^5s^2U34) and yeast tRNA^{Phe} were purified using published procedures [13]. Dethiolation of tRNA^{Lys3} was performed by H_2O_2 treatment to convert the modified 2-thiouridine S34 into mcm^5U34 [6]. The tRNAs were 3′-end-labelled with [^{32}P]pCp as described by Bruce and Uhlenbeck [14]. ONOOK was prepared as previously described [4]; briefly: 10 ml of 1.2 M HCl was added to a stirring solution of 20 ml 0.6 M $NaNO_2$-0.9 M H_2O_2. After 5 s, 10 ml of a solution containing 1.8 M KOH and 400 μl diethylenetriaminepentaacetic acid (DTPA) were supplemented to yield a stable solution of ONOOK. Unreacted H_2O_2 was disproportionated with a platinum mesh electrode. The yellow solution was stored at −80°C and thawed on ice prior to the probing experiments. Procedure for RNA probing: 5 pmol of tRNA were pre-incubated for 10 min at 37°C in a reaction buffer containing 200 mM sodium cacodylate (pH 7.0), 50 mM NaCl and 10 mM $MgCl_2$. 2 μl of a 90 mM ONOOK stock solution were added to 20 μl samples. tRNA was incubated for 15 s at 37°C followed by precipitation with 0.1 vols. of 3 M NaOAc (pH 6) and 3 vols. of ethanol. Samples were washed once with 80% ethanol, redissolved in 80% formamide and loaded on the gel. RNA cleavage with ONOOK was most efficient in cacodylate buffers while reactivity is markedly reduced when using Tris or HEPES. Probing experiments can be performed between pH 6 and 8. The buffer capacity must be sufficiently high to prevent changes of the pH in the sample solution. Final concentrations for [Fe(EDTA)]$^{2-}$ probing experiments were as follows: 1 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_20$, 2 mM EDTA, 0.05% H_2O_2 and 5 mM DTT. The reaction was allowed to proceed for 10 min at 37°C and stopped by adding 5 μl of a 100 mM thiourea solution. Samples were precipitated and loaded on the gel as described.

3. Results and discussion

In order to compare the cleavage specificity of ONOOK and [Fe(EDTA)]$^{2-}$ on structured RNA molecules, we used yeast tRNA^{Phe} and rabbit tRNA^{Lys3} as model systems. Fig. 2 shows comparison of cleavage profiles of 3′-end-labelled tRNA^{Phe} and tRNA^{Lys3} when probed with [Fe(EDTA)]$^{2-}$ and ONOOK, respectively. Both reagents are capable of

*Corresponding author.

PII S0014-5793(96)00662-X

cleaving RNA independent of sequence and secondary structure. Cleaved RNA fragments migrate slightly faster than corresponding fragments generated by partial alkaline hydrolysis which indicates the presence of 5'-phosphate termini. Such reaction products are expected when strand scission occurs via oxidative attack at the sugar moiety. Although ONOOH, the active species from which hydroxyl radicals are generated, is uncharged and significantly smaller than $[Fe(EDTA)]^{2-}$, the protection pattern for the two tRNAs studied is very similar. In agreement with previous reports [3,5], the strongest protection is seen at positions 18, 46, 48, 58, 59 and 60 and additionally at positions 8 and 9. The uncleaved nucleotides are centered around the hinge of the L-shaped tRNA where extensive tertiary interactions stabilize the structure. Protection of Y37 in the anticodon-loop of $tRNA^{Phe}$, observed when probing either with ONOOK or $[Fe(EDTA)]^{2-}$, has been attributed to the modification of this residue [5].

The only obvious difference in reactivity, which must be assigned to the different chemical nature of both reagents, is found at position S34 (mcm^5s^2U) of $tRNA^{Lys3}$. This residue shows hyperreactivity when probed with ONOOK, while its cleavage with $[Fe(EDTA)]^{2-}$ is only slightly enhanced. The intensities of all other bands are therefore little weaker compared with those from the $[Fe(EDTA)]^{2-}$ experiment, in order to ensure 'single hit' conditions. The observation that the ONOOK mediated strand scission at S34 appears at least an order of magnitude stronger compared to those cuts located in the other regions of the tRNA suggests the existence of different reaction pathways. Hyperreactivity is not observed when probing dethiolated $tRNA^{Lys3}$, the modified uridine of which has been identified as mcm^5U [6] indicating that the sulfur atom of the base is crucial for the enhanced cleavage (data not shown). The ONOOK-induced strand scission at S34 is reminiscent of the dethiolation reaction itself, which is performed in the presence of hydrogen peroxide [6,7]. We suggest that both reactions, namely the particular cleavage with ONOOK and conversion of thiouridine into uridine with hydrogenperoxide, may initially require nucleophilic attack of a peroxo-species at the thiocarbonyl group of S34. The peroxonitrite anion itself and the hydrogenperoxide anion,

Scheme 1:

$$[Fe(EDTA)]^{2-} + H_2O_2 \rightarrow [Fe(EDTA)]^{1-} + \mathbf{OH\cdot} + OH^-$$

Scheme 2:

$$ONOO^-K^+ + H_2O \rightarrow ONOOH + K^+ + OH^- \quad (1)$$

$$ONOOH \rightarrow NO_2\cdot + \mathbf{OH\cdot} \quad (2)$$

$$2\,NO_2\cdot \rightarrow N_2O_4 \quad (3)$$

$$N_2O_4 + H_2O \rightarrow NO_3^- + NO_2^- + 2\,H^+ \quad (4)$$

Fig. 1. Generation of hydroxyl radicals (scheme 1) via the Fenton-like reaction during oxidation of chelated Fe^{2+} in the presence of hydrogen peroxide and (scheme 2) by decomposition of peroxonitrous acid in aqueous solution at neutral pH (4). Protonation of the peroxonitrite anion (Eq. 1) is followed by the dissociation of its conjugate acid thereby generating hydroxyl radicals and nitrogen dioxide (Eqs. 2,3), which disproportiates to form nitrite and nitrate (Eq. 4).

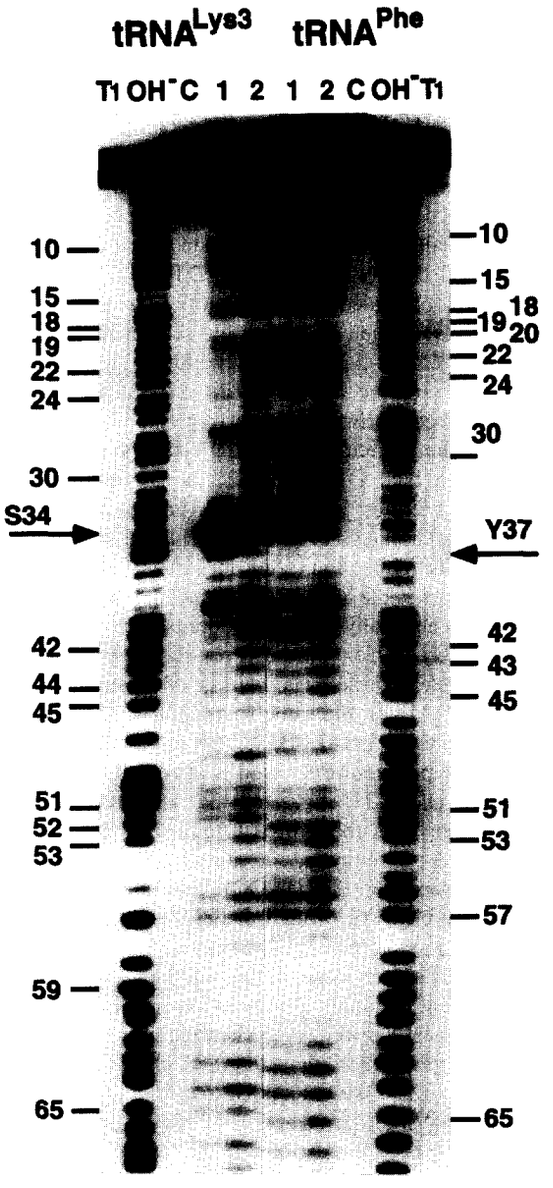

Fig. 2. Cleavage pattern of tRNA applying either ONOOK or $[Fe(EDTA)]^{2-}$. The autoradiograph shows a 10% polyacrylamide gel of 3ϕ-end-labelled rabbit $tRNA^{Lys3}$ (left) and yeast $tRNA^{Phe}$ (right): T1, partial RNase T1 digestion; OH^-, partial alkaline hydrolysis; C, control incubations with reaction buffer only; 1, ONOOK treatment; 2, $[Fe(EDTA)]^{2-}$ treatment.

respectively, are putative candidates for the proposed mechanism.

However, the difference in reactivity between ONOOK and $[Fe(EDTA)]^{2-}$ at the modified thiouridine of $tRNA^{Lys3}$ is an exception. The fact that all other residues show similar reactivities with both reagents strongly support the view that access of the commonly generated hydroxyl radical, rather than the access of its source, determines specificity for strand scission. This interpretation is not undisputed: Zhong and Kallenbach [8] have recently reported that 'subtle differences' in cleavage profiles can be observed when yeast $tRNA^{Phe}$ is probed with $[Fe(EDTA)]^{2-}$ and the neutral metal complex [Fe(EDTA)], respectively, suggesting that at least the charge of the hydroxyl radical source affects accessibility. Different

reactivities of the negatively and the uncharged metal complexes were also detected when probing a four-arm branched DNA complex [9]. In our experience, small differences in reactivity are within the accuracy of the method when probing tRNA either with $[Fe(EDTA)]^{2-}$ or ONOOK.

The nature of the active species itself, which is directly involved in strand scission of DNA or RNA, is also a matter of ongoing discussion. Strong oxidants, chemically distinct from hydroxyl radicals, could also participate in the cleavage reactions mediated by ONOOK or $[Fe(EDTA)]^{2-}$. For example, Sawyer et al. [10] argued that a high-valent iron-oxo complex might be involved when using Fenton-like reactions. The best evidence, suggesting that hydroxyl radicals generated from $[Fe(EDTA)]^{2-}$ are the active species responsible for strand scission, has been provided by Pogozelski et al. [1]. They showed that the particular sinusoidal cleavage pattern on A-tracks within a bent DNA fragment is essentially the same when applying either $[Fe(EDTA)]^{2-}$ or γ-rays which give rise to hydroxyl radicals in aqueous solutions in the absence of heavy metals. This observation indicates that a commonly generated species is the active one. The alternative explanation that putative chemically distinct oxidants generated by these methods, namely hydroxyl radicals produced by γ-rays and an iron-oxo complex formed via Fenton chemistry, would approach the DNA with the same specificity is rather unlikely.

The nature of the active species, generated during decomposition of the peroxonitrite anion is also controversially discussed. Pryor et al. [11] suggested that an activated form of ONOOH rather than the hydroxyl radical itself is the active species. In analogy, following the same reasoning as discussed above, our observation that ONOOK and $[Fe(EDTA)]^{2-}$ generate the same cleavage profile on tRNAs supports the view that the commonly generated hydroxy radical is responsible for strand scission. It seems rather unlikely that chemically entirely different oxidants produced by $[Fe(EDTA)]^{2-}$ and ONOOK can generate the same cleavage pattern on tRNA.

Our data show that the different chemical nature of $[Fe(EDTA)]^{2-}$ and ONOOK is of minor importance when probing the higher order structure of tRNA, indicating that both reagents can be used to define the 'inside' and the 'outside' of structured RNA in general. This conclusion might not be correct, if RNA-protein complexes are analyzed. Hüttenhofer and Noller [5] observed that tRNA bound to ribosome remains uncleaved by $[Fe(EDTA)]^{2-}$ generated hydroxyl radicals at most positions. They explained this observation by the particular structure of the ribosome preventing access of the hydroxyl radical generating reagent, namely $[Fe(EDTA)]^{2-}$ to the bound tRNA. Whether this is correct, or whether the observed inaccessibility is due to specific interactions of tRNA with the ribosome could be clarified by using a different hydroxyl radical generating reagent, namely ONOOK. Comparative footprinting studies on protein/nucleic acid complexes using either $[Fe(EDTA)]^{2-}$ or ONOOK may help to discriminate between different possible modes of protection against hydroxyl radical attack.

Finally, compared with the Fenton reaction, the use of ONOOK offers several advantages: Reaction time is determined by the fast decomposition of ONOOH (ONOOH has a half-life of 1.9 s at pH 7.4 [12]) and therefore it is not necessary to stop the reaction with any hydroxyl radical scavenger such as thiourea; the very fast reaction may also allow footprinting of kinetically labile RNA/protein complexes or even time-resolved footprinting studies [4]; application is very convenient since a solution of ONOOK is simply added into the sample solution, while treatment with $[Fe(EDTA)]^{2-}$ generally requires pre-mixing of four freshly prepared solutions: $Fe(NH_4)_2(SO_4)_2 \cdot 6H_20$, EDTA, H_2O_2 and ascorbate or DTT.

Acknowledgements: This work was supported by SFB 165 and Fonds der Chemischen Industrie (H.J.G.) and by the French Agence Nationale de Recherches sur le SIDA (ANRS). M.G. would like to thank the European Molecular Biology Organization for providing a short-term fellowship to Strasbourg.

References

[1] Pogozelski, W.K., McNeese, T.J. and Tullius T.D. (1995) J. Am. Chem. Soc. 117, 6428–6433.
[2] Latham, J.A. and Cech, T.R. (1989) Science 245, 276–282.
[3] King, P.A., Anderson, V.E., Edwards, J.O., Gustafson, G., Plumb, R.C. and Suggs J.W. (1992) J. Am. Chem. Soc. 114, 5430–5432.
[4] King, P.A., Jamison, E., Strahs, D. Anderson, V.E. and Brenowitz, M. (1993) Nucleic Acids Res. 21, 2473–2478.
[5] Hüttenhofer, A. and Noller, H.F. (1992) Proc. Natl. Acad. Sci. USA 89, 7851–7855.
[6] Isel, C., Marquet, R., Keith, G., Ehresmann, C. and Ehresmann, B. (1993) J. Biol. Chem. 268, 25269–25272.
[7] Watanabe, K. (1980) Biochemistry 19, 5542–5549.
[8] Zhong, M. and Kallenbach, N.R. (1994) J. Biomol. Struct. Dyn. 11, 901–911.
[9] Lu, M., Guo, Q., Wink, D.J. and Kallenbach N.R. (1990) Nucleic Acids Res. 18, 3333–3337.
[10] Sawyer, D.T., Kang, C., Uobeth, A. and Redman, C.J. (1993) J. Am. Chem. Soc. 115, 5817–5818.
[11] Pryor, W.A., Jin, X. and Squadrito G.L. (1994) Proc. Natl. Acad. Sci. USA 91, 11173–11177.
[12] Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A. and Freeman, B.A. (1990) Proc. Natl. Acad. Sci. USA 87, 1620–1624.
[13] Raba, M., Limburg, K., Burghagen, M., Katze, J.R., Simsek, M., Heckman, J.E., RajBhandary, U.L. and Gross, H.J. (1979) Eur. J. Biochem. 97, 305–318.
[14] Bruce, A.G. and Uhlenbeck, O.C. (1978) Nucleic Acids Res. 5, 3665–3667.