Inhibition of Ribonuclease P Activity by Retinoids*

(Received for publication, February 2, 1998, and in revised form, July 1, 1998)

Evangelia Papadimou†, Sophia Georgiou‡, Dionysios Tsambaos§, and Denis Drainas¶

From the Departments of †Biochemistry and §Dermatology, School of Medicine, University of Patras, 26500 Patras, Greece

The effect of two naturally occurring (retinol and all-trans retinoic acid) and two synthetic (isoretinoin and acitretin) analogs of vitamin A (retinoids) on tRNA biogenesis was investigated employing the RNase P of Dictyostelium discoideum as an in vitro experimental system. RNase P is an ubiquitous and essential enzyme that endonucleolytically cleaves all tRNA precursors to produce the mature 5′ end. All retinoids tested revealed a dose-dependent inhibition of RNase P activity, indicating that these compounds may have a direct effect on tRNA biogenesis. Detailed kinetic analysis showed that all retinoids behave as classical competitive inhibitors. The K<sub>i</sub> values determined were 1475 μM for retinol, 15 μM for all-trans retinoic acid, 20 μM for isoretinoin, and 8.0 μM for acitretin. On the basis of these values acitretin is a 184, 2.5, and 1.9 times more potent inhibitor, as compared with retinol, isoretinoin, and all-trans retinoic acid, respectively. Taking into account that retinoids share no structural similarities to precursor tRNA, it is suggested that their kinetic behavior reflects allosteric interactions of these compounds with hydrophobic site(s) of D. discoideum RNase P.

Retinoids, a group of natural and synthetic analogs of vitamin A, play an essential role in vision, growth, and reproduction as well as exhibiting striking effects on cell proliferation, differentiation, and pattern formation during development (1, 2). The discovery that members of the steroid/thyroid hormone receptor superfamily are nuclear retinoid acid-binding proteins tremendously improves our understanding of the mechanisms that mediate the regulatory action of retinoids on gene expression (3–6). The retinoid receptors are ligand-activated, DNA-binding, trans-acting, transcription factors (7, 8). Due to their ability to regulate cell differentiation and suppress or reverse the malignant phenotype, retinoids have a potential use as chemopreventive and chemotherapeutic agents for all-trans retinoic acid and two synthetic (isotretinoin and acitretin) analogs of vitamin A (retinoids) on tRNA biogenesis. Detailed kinetic analysis showed that all retinoids behave as classical competitive inhibitors. The K<sub>i</sub> values determined were 1475 μM for retinol, 15 μM for all-trans retinoic acid, 20 μM for isoretinoin, and 8.0 μM for acitretin. On the basis of these values acitretin is a 184, 2.5, and 1.9 times more potent inhibitor, as compared with retinol, isoretinoin, and all-trans retinoic acid, respectively. Taking into account that retinoids share no structural similarities to precursor tRNA, it is suggested that their kinetic behavior reflects allosteric interactions of these compounds with hydrophobic site(s) of D. discoideum RNase P.

MATERIALS AND METHODS

Assay for RNase P Activity—Enzyme assays were carried out at 37 °C in 20 μl of buffer D (50 mM Tris/HCl, pH 7.6, 10 mM NH₄Cl, 5 mM MgCl₂, and 5 mM dithiothreitol) containing 2–5 fmol of tRNA substrate (an in vitro labeled transcript of the Schizosaccharomyces pombe tRNA<sup>supSI</sup> gene <i>supSI</i>) and 1.3 μg of protein from the RNase P fraction. Stock solutions of retinoids (see Fig. 1), which were kindly supplied by Roche Hellas S.A. (Athens, Greece), were prepared in 100% Me₂SO. Based on high pressure liquid chromatography (one single peak) and NMR spectroscopy (400 MHz NMR Bruker instrument), all retinoids used in the present study appeared to be highly pure. When retinoids were used, enzyme assays were carried out at 37 °C in 20 μl of buffer D in the presence of 10% Me₂SO. The reactions were stopped by the addition of 5 μl of stop dye (80% formamide, 50 mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanol). Reaction products were resolved on a denaturing 10% polyacrylamide/8 M urea gel and visualized by autoradiography without drying. Activity was quantified by Cerenkov counting of excised gel slices.

Enzyme Purification—Growth of <i>D. discoideum</i> cells (strain AX2 wild type) and cell breakage were essentially carried out as described previously (19). Broken cells were centrifuged for 10 min at 8000 × g. The supernatant was removed and spun at 100,000 × g for 1 h, yielding a 15-ml S-100 fraction. The S-100 fraction was loaded onto a DE 52 column (40 ml; 2.1 cm × 24 cm) that had been equilibrated with AK 50 buffer. The column was washed with the same buffer until the A<sub>280</sub> dropped almost to zero. RNase P was then eluted with a 120-ml linear gradient of 50–300 mM KCl in buffer A at a flow rate of 2 ml/min. Activity was eluted at 130–150 m M KCl. The active fractions were pooled and dialyzed overnight against 4 liters of AK 50 buffer. The RNase P fraction was loaded onto a second DE 52 column (20 ml; 2.1 cm × 24 cm) containing 2–5 fmol of tRNA (an in vitro labeled transcript of the <i>S. pombe</i> tRNA<sup>supSI</sup> gene <i>supSI</i>) and 0.1 units of RNase A from bovine pancreas (Sigma), in the presence of 10% Me₂SO.

* This work was supported in part by the Greek Government (General Secretariat of Research and Technology, Ministry of Development). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 30-61-997746; Fax: 30-61-997690; E-mail: Drainas@med.upatras.gr.

This paper is available on line at http://www.jbc.org
The reactions were stopped by addition of 5 μl of stop dye, and reaction products were resolved on a denaturing 20% polyacrylamide/8 M urea gel and visualized by autoradiography without drying.

RESULTS

The present data show that all retinoids investigated here (Fig. 1) are capable of inhibiting the RNase P activity of D. discoideum. The substrate for RNase P assays was an in vitro labeled transcript of the S. pombe tRNA^Ser^ supS1 (20).

The concentration of all-trans retinoic acid (IC_{50}) at which the product formation is reduced by 50% is equal to 80 μM (Fig. 2, A and B). Although not as effective as all-trans retinoic acid, retinol also inhibited RNase P activity; the IC_{50} was equal to 500 μM (Fig. 2, C and D), respectively. It is obvious from these results that acitretin is the strongest inhibitor among the retinoids tested. It is important to note that RNase P purified through the second DE 52 column was inhibited by retinoids to the same extent as RNase P purified through the S-300 column.

The type of inhibition of D. discoideum RNase P activity by the natural and synthetic retinoids was further elucidated by detailed kinetic analysis. Because the yields of RNase P after gel filtration chromatography are low, for the kinetic analysis we used RNase P fractions obtained from DEAE-cellulose chromatography. Due to the high hydrophobicity of retinoids, all assays were carried out in the presence of 10% Me_2SO. At this concentration, Me_2SO marginally affects (K_i = 4 μM) the catalytic parameters of D. discoideum RNase P. The initial velocity in the presence or absence of retinoids was determined from the initial slopes of time plots (not shown). Fig. 4 shows double reciprocal plots with increasing concentrations of acitretin. The lowest line in Fig. 4 represents the data obtained in the absence

---

*Fig. 1. Chemical structures of retinoids. A, all-trans retinoic acid; B, retinol; C, isotretinoin; D, acitretin.*

*Fig. 2. Dose response effect of all-trans retinoic acid (A and B) and retinol (C and D). A and C, autoradiograms of the cleavage of the supS1 precursor by RNase P in the presence of all-trans retinoic acid (A) and retinol (C). A, lane 1, supS1 alone; lane 2, control; lanes 3–8, incubated RNase P in the presence of 0.01, 0.05, 0.1, 0.2, 0.4, and 0.5 mM all-trans retinoic acid, respectively. C, lane 1, supS1 alone; lane 2, control; lanes 3–8, incubated RNase P in the presence of 0.5, 1, 2, 4, 5, and 10 mM retinol, respectively. B and D, RNase P activity as a function of the logarithm of increasing concentrations of all-trans retinoic acid (B) and retinol (D).*

*Fig. 3. Dose response effect of isotretinoin (A and B) and acitretin (C and D). A and C, autoradiograms of the cleavage of the supS1 precursor by RNase P in the presence of isotretinoin (A) and acitretin (C). A, lane 1, supS1 alone; lane 2, control; lanes 3–8, incubated RNase P in the presence of 0.01, 0.05, 0.1, 0.2, 0.4, and 0.5 mM isotretinoin, respectively. C, lane 1, supS1 alone; lane 2, control; lanes 3–8, incubated RNase P in the presence of 0.001, 0.01, 0.02, 0.04, 0.08, and 0.1 mM acitretin, respectively. B and D, RNase P activity as a function of the logarithm of increasing concentrations of isotretinoin (B) and acitretin (D).*
of inhibitor and MeSO (control). The calculated apparent $K_m$ ($K_m,_{app}$) and the apparent $V_{max}$ ($V_{max,_{app}}$) values from this plot are 240 nM and 3 pmol/min, respectively. These values are in agreement with previous values reported from our laboratory (19). In the presence of 10% MeSO, without inhibitor minus MeSO; $\text{\textcopyright}$, without inhibitor plus MeSO; $\text{\textbullet}$, with acitretin at 15 nM; $\triangle$, with acitretin at 25 nM; $\text{\textbullet}$, with acitretin at 30 nM; $\text{\textbullet}$, with acitretin at 50 nM; $\text{\textbullet}$, with acitretin at 75 nM. Top panel, replot of the slopes of the double reciprocal lines versus inhibitor ($I$) concentrations.

**DISCUSSION**

The study of the action of retinoids is of essential importance because these compounds are involved in gene expression, cell growth, and differentiation (1, 2), represent the drugs of choice for a wide spectrum of severe and recalcitrant skin disorders (12–14), and are capable of reversing or suppressing carcinogenesis in many tissues (9–11).

Retinoids act through binding to nuclear receptors that belong to the steroid/thyroid hormone superfamily. It has been proposed that target genes are regulated by these receptors that act as transcription factors activated by retinoids (3–6). However, there is also evidence indicating that retinoid action can be
mediated through mechanisms not involving the retinoid nuclear receptors (22, 23). In the present study it is demonstrated that the important ribosome RNase P isolated from D. discoideum is competitively inhibited by natural and synthetic retinoids.

All retinoids tested revealed a dose-dependent inhibition of RNase P activity, indicating that these compounds may have a direct effect on tRNA biogenesis. Detailed kinetic analysis showed that the type of inhibition of retinol, all-trans retinoic acid, isotretinoin, and acitretin is simply competitive. According to this finding we could assign the potency of inhibitors solely on the basis of the $K_i$ value. Thus, acitretin is 184, 2.5, and 1.9 times more potent than retinol, isotretinoin, and all-trans retinoic acid, respectively.

An interesting observation is that substitution of the CH$_3$O group for hydrogen in the P position of the aromatic ring of the parent compound (all-trans retinoic acid) (Fig. 1) enhances the inhibitory potency of the retinoid. Because the CH$_3$O group increases the hydrophobicity of acitretin, it is possible that this compound may fit better into hydrophobic site(s) of the RNase P. It has been suggested that the low buoyant density of RNase P in Cs$_2$SO$_4$ gradients (19) can be attributed to the occurrence of an unidentified component such as a fatty acid or lipid. This hypothesis is consistent with the existence of hydrophobic site(s) on RNase P. On the other hand, retinol, which is a very hydrophilic molecule, cannot be accommodated into the active site(s) on RNase P. On the other hand, retinol, which is a very hydrophilic molecule, cannot be accommodated into the active site(s) on RNase P (24). This finding results from retinoids titrating to allosteric inhibition sites of the enzyme. Finally, it is possible that the inhibition of RNase P could have been caused by a retinoid-specific receptor complex; the receptor could have been co-eluted with RNase P activity during the purification procedure. However, this hypothesis can be ruled out, because RNase P activity during the purification through the Sephacryl S-300 column elutes with the void volume of the column, with the enzyme behaving as a protein with very high molecular mass (see "Materials and Methods"), whereas retinoid acid receptors, if present in the extract, will elute in later fractions even apart from the RNase P activity, because of their low molecular mass (<50 kDa) (3).

Furthermore, it could be speculated that the inhibition of RNase P activity results from retinoids titrating out a co-activator component from RNase P, for example a bivalent cation like Mg$^{2+}$; nevertheless, this possibility is definitely ruled out because in this case the inhibition pattern is of partial noncompetitive type. Moreover, other types of RNase P co-activators have not been reported so far.

Acknowledgment—We thank Dr. D. L. Kalpaxis for critical reading of the manuscript.

REFERENCES

1. Tsambou, D., and Zimmerman, B. (1991) Effects of Synthetic Retinoids on Cellular Systems in Poriassia (Roennigk, H. H., and Maibach, H. I., eds) pp. 659–707, Marcel Dekker Inc., New York.
2. Ross, A. C. (1993) PAFACER J. 7, 317–327.
3. DeLuca, L. M. (1991) PAFACER J. S, 2924–2933.
4. van der Saag, P. T. (1996) Eur. J. Cell. Biochem. 50, Suppl. 3: 24–28.
5. Botling, J., Castro, D. S., Oberg, F., Nilsson, K., and Perlmann, T. (1997) J. Biol. Chem. 272, 9443–9449.
6. Leid, M., Kastner, P., and Chambon, P. (1992) Trends Biochem. Sci. 17, 427–433.
7. Beato, M. (1989) Cell. 56, 335–344.
8. Minucci, S., Leid, M., Toyama, R., Saint-Jennet, J. P., Peterson, V. J., Horn, V., Ishmael, J. E., Bhattacharyya, N., Dey, A., Dawid, I. B., and Ozato, K. (1997) Mol. Cell. Biol. 17, 644–655.
9. Lippman, S. M., Heyman, R. A., Kurie, J. M., Benner, S. E., and Hong, W. K. (1995) J. Cell. Biochem. 22, Suppl. 1–10.
10. Muidi J. R. (1996) Cancer Treat. Res. 870, 305–342.
11. Sankaranarayanan R., and Mathew, B. (1996) J. Biochem. 324, 357–366.
12. Wieder J. M., and Lowe, N. J. (1995) Dermatol. 314, 1023–1029.
13. Pliewg, G., Albrecht, G., Henz, B. M., Meigel, W., Schopf, E., and Stadler, R. (1995) Hum. 48, 881–885.
14. Darr, S. C., Brown, J. W., and Pace, N. R. (1992) Trends Biochem. Sci. 17, 178–182.
15. Guerrier-Takada, C., Gardiner, T. Marsh, K., Pace, N., and Altman, S. (1983) Cell 35, 849–857.
16. Symons, R. F. (1994) Curr. Opin. Struct. Biol. 4, 320–330.
17. Drainas, D. (1996) Mol. Biol. Rep. 22, 135–138.
18. Stathopoulos, C., Kalpaxis, D. L., and Drainas, D. (1995) Eur. J. Biochem. 228, 776–800.
19. Drainas, D., Zimmerman, S., Willis, I., and Soll, D. (1989) FEBS Lett. 251, 258–262.
20. Segel, I. H. (1977) Enzyme kinetics, p. 110, John Wiley & Sons, Inc., New York.
21. Chao, W. R., Hobbs, P. D., Jiang, L., Zhang, X. K., Zheng, Y., Wu, Q., Shroot, B., and Dawson, M. I. (1997) Cancer Lett. 115, 1–7.
22. Hsu, C. A., Rishi, A. K., Su-Li, X., Gerald, T. M., Dawson, M. I., Schiffer, C., Reichert, V., Shroot, B., Poirier, G. C., and Fontana, J. A. (1997) Blood 88, 4470–4479.

TABLE I

Equilibrium constants derived from primary and secondary kinetic plots

| Compound          | $K_i$ (µM) |
|-------------------|-------------|
| Retinol           | 1475.0      |
| All-trans retinoic acid | 15.0       |
| Isotretinoin      | 20.0        |
| Acitretin         | 8.0         |