Stereoconfiguration Markedly Affects the Biochemical and Biological Properties of Phosphorothioate Analogs of 2–5A Core, (A2′p5′)2A∗

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The diester bonds of phosphorothioate trimer analogs of (A2′p5′)2A (2–5A core) of the Sₚ stereoconfiguration were found to be extremely stable to hydrolysis by both serum and cellular phosphodiesterases. The corresponding Rₚ isomers, although still more stable than parent ppp(A2′p5′)₂A (2–5A), were significantly more susceptible to enzymatic hydrolysis than were the Sₚ isomers. Utilization of these novel 2–5A trimer isomers containing various combinations of Sₚ or Rₚ configurations at the internucleotidic phosphorothioate linkages revealed a further specificity of this enzymatic hydrolysis. Thus, the stereoconfiguration of the bond adjacent to the one undergoing hydrolysis influenced the rate of enzymatic hydrolysis, as well as did the chain length of the oligomer. The most stable trimer analog, which contained both internucleotide phosphorothioate linkages of the Sₚ configuration, had a half-life of 30 days in serum, which is a 1500-fold increase over that of parent 2–5A core. This is the first report on biochemical stability of an oligonucleotide containing more than one phosphorothioate linkage of the Sₚ configuration and is the first demonstration that a phosphorothioate internucleotide bond of the Sₚ configuration can increase the enzymatic stability of an adjacent phosphorothioate bond. In marked contrast to all previous 2–5A core analogs of increased stability, the activity (antiproliferative and antiviral) of the stable phosphorothioate 2–5A core analogs was obtained with the intact trimer, i.e., it was not attributed to antimetabolite degradation products.

Interferon (α, β, or γ) treatment of many cells in culture results in a multitude of biochemical changes, including the induction of several proteins, and ultimately results in the establishment of an antiviral state, inhibition of cell proliferation, and, in certain relevant cells, immunomodulatory effects (reviewed in Stewart, 1981). One of the proteins induced after interferon treatment is an enzyme, 2–5A′ synthetase, which is activated upon binding to double-stranded RNA (Baglioni et al., 1981b; Torrence et al., 1981). The activated enzyme polymerizes ATP into a series of 2′–5′-linked adenylyl oligonucleotides containing a triphosphate at the 5′ terminus (Ball and White, 1978; Kerr and Brown, 1978; and reviewed in Baglioni, 1979; Lengyel, 1982; and Torrence, 1982). Oligomers containing a 5′-di- or triphosphate and three or more monomer units bind to, and subsequently reversely activate, an endogenous or sometimes interferon-induced (Jackson et al., 1983a, 1983b; Silverman et al., 1983a) endoribonuclease (Baglioni et al., 1978; Ball and White, 1979; Clemens and Williams, 1978; Eppstein et al., 1979; Eppstein and Samuel, 1978; Farrell et al., 1978; Ratner et al., 1978; Williams et al., 1979a). The activated endonuclease cleaves messenger and ribosomal RNAs (Silverman et al., 1983b; Wreschner et al., 1981), resulting in inhibition of translation. The 2–5A molecules are readily cleaved by cellular phosphodiesterases, and thus their ability to inhibit protein synthesis is transitory (Williams et al., 1978). The 2–5A system has been implicated as contributing to the antiviral state in several cell-virus systems (reviewed in Baglioni, 1978; Lebleu and Content, 1982; Lengyel, 1981, 1982; Revel, 1979; Torrence, 1982). The 2–5A core, which lacks the terminal 5′-phosphates, does not bind to (Knight et al., 1980) or activate the endonuclease (Williams and Kerr, 1978; Eppstein et al., 1983b). Recently, the detection of high concentrations of 2–5A core in intact cells (Kerr et al., 1983; Knight et al., 1980) raises the question of its biological significance. However, meaningful studies have been hampered by its extremely short half-life, and thus a stabilized analog is desirable.

In order to study the 2–5A system, we and others have employed analogs of 2-5A containing modifications primarily on the sugar moiety (Baglioni et al., 1981a; Chapekar and Glazer, 1983; Doetach et al., 1981; Eppstein et al., 1982, 1983a, 1983b; Imai et al., 1982; Lee and Suhadolnik, 1983; Sawai et al., 1983; Suhadolnik et al., 1983), on the base (Drocourt et al., 1982; Torrence et al., 1984), or changing the 2′–5′ phosphodiester linkage to a 3′–5′ linkage (Lesia et al., 1983). Initial studies suggested that analogs of 2–5A core, which contained modified sugar residues and were stabilized to degradation by cellular phosphodiesterases, showed significant antiproliferative and antiviral activities on nonpermeabilized cells, in contrast to the unstable parent 2–5A core. However, it was subsequently demonstrated that all the biological activities of these "stable" 2–5A core analogs were attributable to the antimetabolite degradation products produced as a consequence of hydrolysis by serum enzymes (Chapekar and Glazer, 1983; Eppstein et al., 1983a). We wished to study an analog of 2–5A core that was stabilized both to cellular as well as serum phosphodiesterases and which, even if it were eventually degraded, would not yield antimetabolite nucleosides. Such an analog could provide a handle in studying, in the intact cell system, whether intrinsic biological activities of 2–5A core existed which were unrelated to endonuclease activation. We chose to study phosphorothioate derivatives of 2–5A core as candidates for meeting the above requisites.

It has been determined for some polynucleotide hydrolases that phosphorothioate internucleotide linkages of either the

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1 The abbreviations used are: 2–5A, ppp(A2′p5′)₂A; 2–5A core, (A2′p5′)₂A.
phosphodiester bond undergoing hydrolysis, the phosphodiester bond of the intact stabilized 2-5A trimer core.

We have utilized novel chemically synthesized 2-5A analogs which are modified at the 2'-5' phosphodiester linkage by the substitution of a sulfur for an oxygen (Nelson et al., 1983a). This substituted phosphorous is chiral and exists in either $R_P$ or $S_P$ stereoconfiguration. Consequently, the resulting core analogs (i.e., lacking the 5'-triphosphate) of trimers 2-5A core can exist as four stereoisomers ($R_R, R_S, S_R, S_S$). We have utilized this novel set of analogs to analyze the specificity of the cellular and serum phosphodiesterases that degrade the 2-5A species via hydrolysis of the phosphodiester bond. We report that, in addition to the stereoconfiguration preference of the phosphodiester bond undergoing hydrolysis, the phosphodiesterases also recognize the configuration of the phosphodiester bond adjacent to the bond undergoing hydrolysis. The result is that the analog most stable to cleavage contains the $S_P$ configuration at both phosphodiester bonds. The biological activity of these novel stable 2-5A core analogs on intact cells is also determined. For the first time, both antiproliferative and antiviral activities were obtained which are attributable to the intact stabilized 2-5A trimer core.

MATERIALS AND METHODS AND RESULTS

DISCUSSION

Previous studies employing polynucleotides containing the phosphorothioate diester linkage have been limited to enzymatically synthesized polymers with the $R_P$ stereoconfiguration. To date, all polymers studied utilize only the nucleoside 5'-O-(1-thiophosphates) of the $S_P$ stereoconfiguration and result in inversion of configuration at the phosphorus in their polymerization (Eckstein, 1985; Lee and Suhadolnik, 1985). Our present studies utilizing chemically synthesized trinucleotides allow us to examine for the first time the influence of adjacent $S_P$ versus $R_P$ stereoconfigurations on the enzymatic stability at the phosphorothioate bond.

The stability of 2-5A analogs to phosphodiesterase-mediated degradation is dependent on both the structure of the sugar moiety of the adenylic unit (Epptstein et al., 1982, 1983a, 1983b, 1985; Sawai et al., 1983) as well as the structure of the phosphodiester linkage (Lee and Suhadolnik, 1985; Nelson et al., 1984). Modifications that serve to "block" the 2' to 3' end of the 2-5A trimer also significantly increase its stability (Baglioni et al., 1981b; Bayard et al., 1984; Imai et al., 1982).

We now show that in addition, the stereoconfiguration of a modified phosphodiester linkage significantly affects its enzymatic stability, as also does the substrate chain length. Thus, maximally productive interaction of both cellular and serum phosphodiesterases with 2-5A molecules has the appearance of involving multiple "subsites" on the enzyme such as described for other hydrolases (Allen, 1980). The stereoconfiguration of the phosphorothioate linkage increased the stability to all phosphodiesterases tested, with the $S_P$ conformation significantly more stable than the $R_P$ (Table 1, Fig. 2).

Most interestingly, this same effect was also observed with the phosphorothioate linkage adjacent to the one undergoing hydrolysis. That is, the trimer $S_PS_P$ was more stable than the trimer $R_S S_P$ (Table 1, Fig. 2), even though in both cases the bond undergoing hydrolysis was of identical $S_P$ configuration.

The cellular phosphodiesterases showed more discrimination as to substrate specificity than did the serum enzymes, as illustrated by the greater than 300-fold increased stability of the phosphorothioate dimer $R_PS_S$ over the parent 2-5A trimer in cell extracts versus only a 5-fold stability differential for the same molecules in serum (Table 1 and Fig. 4). These results are in parallel with our previous findings of the reduced stability of xyleadenosine 2-5A analogs to serum versus cellular enzymes (Epptstein et al., 1983a).

Previous studies employing 2-5A core analogs of increased metabolic stability have shown marked antiviral and antiproliferative activities. However, upon analysis, it was confirmed that all these activities were due to the antimetabolite effects of nucleoside analogs obtained as degradation products, both for the cordycepin 2-5A cores (Chapekar and Glazer, 1983) and the xyleadenosine 2-5A core analogs (Epptstein et al., 1983a). Accordingly, the antimetabolite activities were markedly increased in the presence of the adenosine deaminase inhibitors coformycin or deoxycoformycin. The availability now for the first time of 2-5A trimer core analogs that are extremely stable to both serum as well as cellular phosphodiesterases and which would not yield antimetabolite nucleosides even if they were to be degraded allowed us to assess what biological activity (if any) could be attributed to an intact 2-5A core species after treatment of nonpermeabilized cells. Anticellular and antiviral activities were obtained, but only with 25-50 $\mu$m phosphorothioate 2-5A core analogs. The more stable $S_P$ isomers were more active than the $R_P$ isomers. As predicted, ribosomal RNA analysis of cells treated either with the phosphorothioate analogs of 2-5A or with parent 2-5A core (Epptstein et al., 1983b) did not show significant degradation of the RNA such as is obtained when parent 2-5A 5'-triphosphate is introduced into cells via calcium phosphate coprecipitation. Thus, the activity obtained with the 2-5A core analogs does not appear to be due to activation of the 2-5A-dependent endonuclease.

We can for the first time conclude that antiviral and antiproliferative activities are obtained with intact 2-5A core species on nonpermeabilized cells. In contrast to previous studies, these activities are not mediated through various degradation products. The activity of 2-5A cores, which do not activate the endonuclease in these nonpermeabilized cells, is much lower than the activity of 5'-triphosphate 2-5A in cell-free extracts or after calcium-phosphate precipitation onto intact cells. However, it is not known how much (if any) of these externally added 2-5A core analogs actually penetrate into the cell. Our present results do not rule out an effect of 2-5A cores at the cell surface, for example by interacting with...
adenosine or ADP receptors. In another system, however, we have determined that phosphorothioate 2′-5′A cores do not inhibit ADP-induced platelet aggregation, such as is observed with adenosine or AMP.3

Lee and Suhadolnik (1985) recently reported that enzymatically synthesized 5′-triphosphate phosphorothioate 2′-5′A trimmer and tetramer analogs, which contained the Rp configuration at the 2′-5′ phosphorothioate bonds, were more potent than parent 2′-5′A 5′-triphosphate in inhibiting protein synthesis in cell extracts. The 5′-triphosphate of the more stable Sp isomers is not yet available, as it cannot be prepared enzymatically and the chemical synthesis is not trivial. When it is available, future studies will determine what activities can be attributed to the 5′-triphosphates of the more stable 2′-5′ phosphorothioate Sp isomers.

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Stereoc configuration Markedly Affects the Biochemical and Biological Properties of Phosphorothioate Analogs of 2-5A Core

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MATERIALS AND METHODS

Materials. Phosphorothioate 2-5A dimer (R, S, and S) and trimer analogs (pool RpRp = RpRp = SSp, pool SpSp = SpSp = RpS, and pool SpSp = SpSp = SpS) configurations were chemically synthesized and purified at Sante Research as previously reported (Nelson & al., 1984). Parent 2-5A dimer ([32P]P5A) and trimer ([32P]P5P) were isomerically pure and were from C. H. W. MacMillan, Chemistry Department, Boehringer Mannheim (Indianapolis, IN). Enzyme snake venom phosphodiesterase I (SVPO) was from Boehringer-Mannheim (Indianapolis, IN). 3T3 fibroblasts were from A. S. Kelley, National Institutes of Health, Bethesda, Maryland. Vero cells were obtained from C. A. M. Bockmann, University of California, Santa Barbara, CA. Herpes simplex virus type 1 (HSV-1, strain R) was obtained through the collection of the American Type Culture Collection (Philadelphia, PA). 

Stability of 2-5A Analogs. The stability of 2-5A core analogs to enzymatic degradation by serum or cellular phosphodiesterases was determined as described (Epstein & al., 1985), except that incubations were at 37°C. Half-lives were calculated (Epstein & al., 1985) after incubation of 100 µM substrate in either media containing 10% calf serum; or in HPLC separations were done on a Whatman Partisil 10, 1.5 mlfmin with a mobile phase of 250 mM ammonium phosphate (pH 7.0) and a linear gradient of 0-25% methanol.

Phosphorothioate Analogs for the assay were purified to 100% by preparative HPLC of the phosphorothioate 2-SA core analogs. The purity of the phoshothioate 2-SA core analogs was determined by reducing the disulfide bridge with sodium borohydride, and the chemical addition of the [35S]methionine as described (Eppstein & al., 1982). 

Biological Assay. Serum-stimulated mitogenesis of Swiss 3T3 cells, previously described (Eppstein & al., 1983), was determined by plaque reduction assay in Vero cells as described (Eppstein & al., 1985).

Table 1: Stabilities of Phosphorothioate 2-5A Core Analogs

| Half-Life (Days), 37°C | Serum | 10% Cell Extract |
|-----------------------|-------|-----------------|
| 2-5A Core Analogs     |       |                 |
| Phosphorothioate       |       |                 |
| Dimer R, isomer        | 1     | 2               |
| Dimer S, isomer        | 0.2   | 0.5             |
| Trimer RpRp mixture    | 0.1   | <0.5            |
| Trimer SSp SpSp        | 0.1   | 0.2             |
| Parent 2-5A Core       | 0.2   | 0.009           |

Due to the extreme stability of the S configuration, very little degradation of some isomers was observed (see Table 1). The activity of the phosphodiesterases in the cell-free extract, however, began to decrease (as monitored by degradation of the parent 2-5A core) and after 2 days incubation the activity was only half as potent as after 1 day incubation. 

Stability determinations in cell extracts were accordingly limited to 2 days incubation. In a typical experiment the activity of the phosphodiesterases was determined in cell extracts and in serum to be approximately 0.009 and 0.009, respectively.

The half-life determinations to serum were based on a 4-day incubation, and are accordingly more accurate for the very stable analogs.
Phosphorothioate Analogs of 2-5A

Loss of ability of cellular phosphodiesterases to degrade 2-5A after preincubation at 37°C. L cell S-10 extracts were preincubated at 37°C for 0, 1, 2, or 4 days before addition of 100 μM parent 2-5A core and further incubation for up to 24 min to determine the half-life of the 2-5A.

The phosphorothioate dimer 2-5A analogs were found to be considerably more stable than were the corresponding trimer analogs (Table 1). This also was true for the parent 2-5A core oligomers when analyzed with cell extracts (Fig. 4). In the presence of serum enzymes, however, the parent 2-5A dimer and trimer analogs were of comparable stability, which was approximately twice that of the tetramer species. Overall, the parent 2-5A analogs were 10-24 times more stable in cell extracts than in serum.

The trimer S mixture showed greater antiviral activity against HSV-1 than did the trimer R mixture at 50 μM, while parent 2-5A core showed no activity (Table 2). No ribosomal RNA degradation was detected after treatment of cells with these 2-5A core analogs (not shown).

Table 2

| % Inhibition | Mitogenesis | HSV-1 | Replication |
|--------------|-------------|------|-------------|
| Media Control | 0 | 0 | 0 |
| Parent 2-5A Core | 25 μM | -30 | 60 | - |
| 50 μM | 70 | 50 | 0 |
| Phosphorothioate 2-5A Core (Trimer S mixture) | 25 μM | 15 | 30 | - |
| 50 μM | 65 | 35 | 35 |
| (Trimer R mixture) | 25 μM | 2 | 0 | - |
| 50 μM | 50 | 40 | 20 |
| Interferon | 100 U/ml | 25 | 25 | - |

%Cell viability reduced by 20%.

Interferon was NIH reference standard of mouse fibroblast interferon (α + β), #HCF-904-511.

Influence of substrate chain length on 2-5A phosphodiesterase activity. The half-lives of parent 2-5A dimer, trimer, or tetramer cores were determined as described in Materials and Methods. L cell S-10 extracts (upper panel) or in 10% calf serum (lower panel). α, dimer; β, trimer; γ, tetramer.

Influence of substrate chain length on 2-5A phosphodiesterase activity. The half-lives of parent 2-5A dimer, trimer, or tetramer cores were determined as described in Materials and Methods. L cell S-10 extracts (upper panel) or in 10% calf serum (lower panel). α, dimer; β, trimer; γ, tetramer.