Preparation, Characterization, and Use of an Antiserum Specific for 2',5'-Oligoadenylates*

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Periodate-oxidized ppp(A2'p)3A was coupled in high yield to an acyl hydrazide derivative of bovine serum albumin and the conjugate was used to immunize rabbits. Several potent and specific antisera were obtained, and one was tested extensively. Fifty per cent of the bound probe ppp(A2'p)3A3'[32P]pCp was displaced by compounds containing the moiety -pA2'pA- at concentrations of 20–60 nm and by compounds containing the moiety -p(A2'p)2A- at 1 nm. The 3',5'-oligoadenylate (A3'p)3A was bound more than 10,000 times less tightly than (A2'p)2A. The antiserum can be used in competition assays in which the amount of an antibody-probe complex is measured after binding to Millipore filters or to polystyrene beads. These assays allow the clear detection of as little as 20 fmol (20 nl of 1 mM) of 2',5'-oligoadenylates.

The level of 2-5A synthetase increases markedly when most cells are treated with interferon. This enzyme, which catalyzes the formation of 2-5A from ATP when activated by double-stranded RNA, is one of a set of three proteins which are involved in the degradation of single-stranded RNAs in interferon-treated cells. 2-5A activates an endonuclease which is involved in the degradation of single-stranded RNAs in interferon-treated cells. 2-5A activates an endonuclease which catalyzes the formation of 2-5A from ATP when activated by double-stranded RNA, and a 2' → 5'-phosphodiesterase degrades 2-5A, allowing the system to be turned off (Lengyl, 1982). Both 2-5A itself and 2-5A "cores," which lack the 5'-terminal triphosphate, have been observed in interferon-treated cells infected with encephalomyocarditis virus (Williams et al., 1979). 2-5A synthetase is present in many types of animal cells and its levels vary with the cell cycle (Krishnan and Baglioni, 1981; Creasey et al., 1983) and with hormone treatment (Stark et al., 1979), suggesting that the 2-5A system functions outside the interferon response and may be involved in the normal regulation of cellular RNA levels. However, firm evidence for this suggestion is lacking because the mere presence of 2-5A synthetase is not proof that the 2-5A system actually participates in a physiological process. Since the enzyme requires double-stranded RNA (or possibly other, unknown effectors) for activation, we do not know whether enzyme assayed in vitro after binding to exogenous double-stranded RNA was functioning in the cell or, if it was, whether amounts of 2-5A sufficiently high to activate the endonuclease were able to accumulate. To examine further the possible roles of the 2-5A system, it is essential to have direct, sensitive assays for 2-5A and related compounds. We have developed a radioimmunoassay for 2-5A and other 2',5'-oligoadenylates, using antibody made against ppp(A2'p)3A coupled to an acyl hydrazide derivative of bovine serum albumin. This assay complements the radioimmune assays previously developed by Knight et al. (1980, 1981) and Cailla et al. (1982) in which antibodies directed against dephosphorylated forms of 2-5A are used, and the radiobinding assay of Knight et al. (1980, 1981), which employs crude extracts of cells as a source of a binding protein for 2-5A (probably the endonuclease).

EXPERIMENTAL PROCEDURES

Materials—Oligomers of 2-5A were synthesized using poly(I)-poly(C) cellulose (Stark et al., 1979; Wells et al., 1983) and purified on diethylaminoethyl-Sephadex (Martin et al., 1979) using the volatile buffer triethylammonium bicarbonate, pH 7.8, with a gradient from 0.4 to 1.2 M. Alternatively, high pressure liquid chromatography (Brown et al., 1981) was used for some preparations. The 2',5'-oligoadenylates NRppA2'pA, NRpp(A2'p)2A, Rpp(A2'p)2A, and A5'pppp5'(A2'p)2A (Cayley and Kerr, 1982) were generously provided by P. J. Cayley, Imperial Cancer Research Fund, London, England. Other nucleotides and oligonucleotides were obtained from Sigma and P-L Biochemicals.

Coupling of Oxidized 2-5A to Hemocyanin—A mixture of oligomers with average chain length 3–4 was used. A freshly prepared solution of 50 mM sodium metaperiodate, pH 6.1 (12.9 µl, 0.64 µmol) was added to 426 µl of a solution of 3H-labeled 2-5A (1.6 pmol of AMP residues) were mixed with 5 µl of mouse hemocyanin (17.5 mg/ml) in buffer (0.5 mM sodium phosphate, pH 6.9, 0.5 m Hepes-KOH, pH 8.25, or 0.5 m sodium bicarbonate, pH 9.2). A 50–70-fold excess of 2-5A per molecule of hemocyanin was added. NaCNBH3 (3 µl of a 7 mg/ml solution) was added and the reaction mixtures were incubated at 4 °C or at room temperature for 42 h. Reaction yields were calculated by determining the amount of radioactivity bound to hemocyanin after precipitation with trichloroacetic acid.

Coupling of Oxidized 2-5A to a Hydrazide Derivative of Bovine Serum Albumin—The carboxyl groups of albumin were converted to hydrazides by a modification of published procedures (Hoare and Koshland, 1967; Fersht and Sperling, 1973). Hydrazine (15 mM; final concentration, 1 M) was added to an aqueous solution of albumin (83 mg, 1.2 µmol) and then 6 µl HCl (about 14 µmol) was added to bring the pH to 5.25. Ethyl(dimethylaminopropyl)carbodiimide (Sigma, 1.5 µmol) was added and the reaction mixture was incubated at room temperature for 1 h, then placed on a column of Sephadex G-25, 2.4 × 34 cm, and eluted with 20 mM ammonium acetate, pH 7.0. Fractions containing protein were pooled and lyophilized. 3H-labeled ppp(A2'p)3A and 1.0–1.2 eq of sodium metaperiodate (in water, prepared just before use) were incubated on ice, in the dark, for 1 h. The solution was then diluted with water to give a 2-5A

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1 The abbreviations used are: 2-5A, ppp(A2'p)3A; (n ≥ 2); NRpp(A2'p)2A, 2',5'-oligoadenylates with NAD at the 5' terminus; Rpp(A2'p)2A, 2',5'-oligoadenylates with ADP-ribose at the 5' terminus; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
concentration of 0.3–0.5 mM. The oxidized 2-5A (8–10 eq) was added dropwise to an ice-cold, rapidly stirred solution of albumin hydrazide (1 mg/ml) in 0.1 M sodium acetate, pH 4.75. The reaction mixture was incubated at 4 °C overnight. During this time, some of the 2-5A-albumin precipitated. If the oxidized 2-5A is not added to the albumin hydrazide slowly and as a dilute solution, a fraction of the albumin and 2-5A will precipitate, presumably because some molecules of albumin become highly substituted with 2-5A. The reaction yields were 80–90%.

Assays—Assays were done in final volumes of 25 pl, using 1:500 dilution of 
radioactive probe. The probe, obtained by bleeding from an ear vein 9 and 17 days after the fifth injection, was incubated at 4 °C overnight. During this time, some of the 2-5A-oligo-adenylic acid (2',5') was internally transferred to the albumin. After incubation, the conjugates were dialyzed against a Tris-saline buffer and homogenized with an equal volume (1 ml) of Freund’s adjuvant. Complete Freund’s adjuvant was used for the first two injections and incomplete adjuvant was used later. The first three injections were at 2-week intervals, followed by monthly boosts. The sera used for most experiments were obtained by bleeding from an ear vein 9 and 17 days after the fifth injection. The titer of the serum obtained after 17 days was about twice that of the serum obtained after 9 days.

Adsorption—The buffer was 0.1 M Tris-hydrochloride (pH 7.4), 0.45 mM MgCl2, 0.45 mM CaCl2, pH 7.4. Antiserum was diluted with assay buffer containing 0.1% gelatin. (ppiA2`Pp)3A`PpCp (1000–3000 Ci/mmol), prepared as described by Silverman et al. (1981) or purchased from Amersham, was used as the probe in most experiments. 2-5A probes labeled with [3H]ATP or with [32P]ATP also work well, but, since these probes have lower specific activities, the sensitivity is lower. Assays were done in final volumes of 25 p1, using 1:500 to 1:1000 dilutions of antiserum. Standard compounds or unknowns were incubated with antiserum for 1 h at 0 °C. Probe (2500–3000 cpm, diluted in the assay buffer) was added and incubation was continued at 4 °C for 16–18 h. A 24-μl portion of each sample was applied to a Millipore filter (type HA, 0.45 pm) which was already ready to use. During this time, some of the 2-5A was precipitated by trichloroacetic acid. When albumin was applied to a Millipore filter (type HA, 0.45 μm) which was already washed again, and stored in buffer at 4 °C. Each bead carries 150–200 ng of protein A.

The serum binds the 2',5'-linked trimer and tetramer triphosphates tightly, but dimer triphosphate is bound 40 times less well than trimer triphosphate. Removal of the terminal triphosphate weakens binding for the trimer but has a much smaller effect on binding of the tetramer. Several compounds with 2',5'-linked AMP groups bind to the antibody with high affinity. However, oligoadenylates with 3',5'-linkages bind to the antibody more than 10,000 times weaker than the 2',5'-linked oligoadenylates.
times less tightly than the corresponding 2',5'-linked compounds. ATP, ADP, and AMP bind 106-fold less tightly than 2',5'-linked trimer or tetramer triphosphates. The minimum moiety required for moderately tight binding (50% displacement of probe) is a concentration range of about 50-fold, and it is very convenient to read the concentrations of unknowns directly from such curves. The use of this assay to measure concentrations of 2',5'-oligoadenylates in simian virus 40-infected, interferon-treated cells is presented in the accompanying paper (Hersh et al., 1984) and its use in measuring these compounds in extracts of chick oviducts and rat mammary glands is given by Reid et al. (1983).

**DISCUSSION**

Reaction of periodate-oxidized 2',5'-dA with the acyl hydrazide derivative of bovine serum albumin provides a convenient route to conjugates, with high yields and without degradation. This method may be more generally useful for attaching oligonucleotides to proteins than the procedure in which Schiff bases are reduced with a borohydride. Antibodies against a variety of nucleic acids and oligonucleotides have been made using conjugates prepared by coupling the oxidized nucleotides to the lysine ε-amino groups of proteins (Erlander and Beiser, 1964). For example, an AMP-albumin conjugate with 29 AMP residues per albumin was made by reaction with 62 eq of oxidized nucleotide at pH 9-9.5, followed by reduction with NaBH4. Reaction of ApApA, ApApC, and ApUpG under similar conditions yielded conjugates with 8-10 haptenic groups per molecule of albumin (D'Alisa and Erlander, 1974). Reaction of oxidized 7-methyl GMP with albumin at pH 6.5-7.0 in the presence of NaCNBH3 gave a conjugate in about 17% yield (Meredith and Erlander, 1979). These yields are higher than those we obtained using 2,5-A under conditions similar to those used for 7-methyl GMP, but they are lower than our yields for coupling oxidized 2',5'-A to albumin-hydrazide. The antibodies to ApApC and ApUpG (D'Alisa and Erlander, 1974) recognized the mononucleotide attached to the protein in addition to the trinucleotide. Bonavida et al. (1972) made a similar observation with antibodies against several dinucleotides. Since we observed loss of 2',5'-A by β-elimination using a similar coupling procedure, it seems possible that degradation of the trinucleotide conjugate contributed to the cross-reactivity observed. In contrast, antibodies described above in that little displacement of probe was observed in the presence of 100 nM (A2'p)3A.

**Assays—**The filter-binding and bead assays are compared for standard solutions of 2'-5'A in Fig. 2. The standard curves are nearly identical, with half-displacement of the probe at 1.5 nM 2'-5'A for the filter assay or 1.2 nM 2'-5'A for the bead assay. Semilog plots typically give straight lines over a concentration range of about 50-fold, and it is very convenient to read the concentrations of unknowns directly from such curves. The use of this assay to measure concentrations of 2',5'-oligoadenylates in simian virus 40-infected, interferon-treated cells is presented in the accompanying paper (Hersh et al., 1984) and its use in measuring these compounds in extracts of chick oviducts and rat mammary glands is given by Reid et al. (1983).

**FIG. 1. Synthesis of 2-5A-albumin by hydrazide-mediated coupling.**

**FIG. 2. Radioimmune assays for 2-5A.** ø, filter-binding assay; c, bead assay.
against ApApA (D’Alisa et al. 1974) or (A2’p)3A (Knight et al., 1980), both made by the procedure of Erlanger and Beiser (1964), were quite specific for each trimucleotide. Cailla et al. (1982) obtained monoclonal antibodies against A2’pA after coupling succinylated A2’pA to albumin. The reaction of A2’pA with succinic anhydride gave a mixture of products, with the desired diacetyl derivative representing 30% of the total. Coupling oxidized 2-5A to the hydrazide derivative of albumin appears to be the most efficient method for synthesizing 2-5A conjugates.

The antigen we used was made with the tetramer triphosphate ppp(A2’p)4A so that the antibodies would be sensitive to the trimer triphosphate moiety ppp(A2’p)3A, which remains unaltered after periodate oxidation and coupling to the acyl hydrazide. The antibodies obtained are 13-40 times more sensitive to compounds containing the moiety -p(A2’p)2A-than to compounds containing -pA2’pA-. They bind many different 2’,5’-oligoadenylates tightly and they are quite specific for compounds with 2 or more AMP residues linked by 2’,5’-phosphodiester bonds.

When 2-5A synthetase is incubated in vitro with equivalent concentrations of NAD and ATP, the predominant products are NRppA2’pA and NRpp(A2’p)2A. The preference of 2-5A synthetase for adding AMP to acceptors other than ATP in vitro is also observed when reactions are performed with RppA (ADP-ribose) or A5’pppp5’A and ATP. It is of great interest to determine whether any of these compounds, or others in which something other than ATP initiates a chain of 2’,5’-oligoadenylates, are synthesized in vivo. The radioimmunosay is as sensitive as the radiobinding assay of Knight et al. (1980, 1981) for detecting ppp(A2’p)3A, NRpp(A2’p)2A, Rpp(A2’p)2A, or A5’pppp5’(A2’p)2A when n is greater than 2. The antibody binds some compounds more tightly (see Table I), making the radioimmunoassay more responsive to such compounds than the radiobinding assay. See also the accompanying paper (Hersh et al., 1984), in which both assays have been applied to samples derived from cells infected with simian virus 40 and treated with interferon.

The antisera against the 2-5A “core” (A2’p)2A provide an assay for 2’,5’-oligoadenylates lacking 5’-phosphates, which is as sensitive as the assay described here or the radiobinding assay. The monoclonal antibodies developed by Cailla et al. (1982) against A2’pA provide the most sensitive assay for 2’,5’-oligoadenylates and are invaluable in this respect. However, in order to measure low levels of 2’,5’-oligoadenylate-5’-oligophosphates with this assay, the samples must first be treated with a phosphatase. Moreover, until the anti-A2’pA sera are tested more widely, it is not clear that they will be useful for detecting 2’,5’-oligoadenylates which have been initiated with something other than ATP. Since a variety of different 2’,5’-oligoadenylates are present in cells and tissues (Reid et al., 1983; Hersh et al., 1984), it is advantageous to have several different assays with different specificities available.

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