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Isolation of Intercalator-Dependent Protein-Linked DNA Strand Cleavage Activity from Cell Nuclei and Identification as Topoisomerase II

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ABSTRACT: DNA intercalating agents such as 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) have previously been found to induce in mammalian cells the formation of protein-associated DNA single- and double-strand breaks. In the current work, an activity characterized by the production of DNA–protein links associated with DNA strand breaks and by stimulation by *m*-AMSA was isolated from L1210 cell nuclei and was shown to be due to topoisomerase II. Nuclei were extracted with 0.35 M NaCl, and the extract was fractionated by gel filtration, DNA–cellulose chromatography, and glycerol gradient centrifugation. A rapid filter binding assay was devised to monitor the fractionation procedure on the basis of DNA–protein linking activity. The active DNA–cellulose fraction contained both topoisomerase I and topoisomerase II whereas the glycerol gradient purified material contained only topoisomerase II activity. The properties of the active material were studied at both stages of purification. *m*-AMSA enhanced the formation of complexes between purified topoisomerase II and SV40 DNA in which the DNA sustained a single- or double-strand cut and the enzyme was covalently linked to the 5′ terminus of the DNA. This action was further enhanced by ATP, as well as by nonhydrolyzable ATP analogues. *m*-AMSA inhibited the topoisomerization and catenation reactions of topoisomerase II, probably because of trapping of the enzyme–DNA complexes. The activity showed a dependence on the type of DNA intercalators used, analogous to what was previously observed in intact cells. *m*-AMSA had no effect on topoisomerase I. The results serve as a basis for the utilization of alkaline elution assays of drug-induced protein-associated DNA strand breaks as a functional measure of topoisomerase II in cells. *m*-AMSA enhanced the formation of DNA strand breaks and DNA–protein links optimally when the concentration of purified extract was low; at high extract concentrations, DNA strand breaks and DNA–protein links occurred even in the absence of *m*-AMSA. The possibility is discussed that DNA binding activity and topoisomerase activity are alternative functional states of the enzyme.

When mammalian cells are treated with certain DNA intercalating agents, such as ellipticine and 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA), DNA strand breaks can be detected which appear to be associated with DNA–protein cross-links (Ross et al., 1978, 1979; Zwelling et al., 1981). Ross et al. (1979) proposed that this effect is due to the trapping of a topoisomerase in a covalent DNA-bound state. The formation of protein-associated DNA strand breaks in cells treated with *m*-AMSA was found to be strongly temperature dependent and to saturate at about 60 000 per cell (Zwelling et al., 1981). Strand breaks of the single-strand and double-strand types occurred at ratios that differed for different intercalating compounds (Zwelling et al., 1982). It was originally proposed that the single- and double-strand breaks were produced by topoisomerase types I and II, stimulated to different degrees by different intercalators (Zwelling et al., 1981).

Filipski & Kohn (1982) demonstrated the formation of intercalator-induced protein-associated DNA strand breaks in isolated mouse L1210 cell nuclei. The reaction was reversible and required Mg^{2+}; it did not require the addition of ATP and was in fact stimulated by nonhydrolyzable ATP analogues (Pommier et al., 1982, 1984a). Filipski et al. (1983) utilized the isolated nuclei system to develop an assay for the responsible enzyme. The endogenous activity in isolated nuclei was found to be removed by extraction with 0.35 M NaCl. The intercalator-dependent formation of DNA strand breaks and DNA–protein cross-links (in one to one proportion) could then be restored by addition of 0.35 M NaCl nuclear extract. The extract was fractionated by gel filtration, and the activity

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was found in a high molecular weight component that was separated from a peak of topoisomerase I activity. In the current work, we have further purified and characterized this activity from mouse L1210 cell nuclei and find it to be a topoisomerase II. We conclude that the protein-associated single-strand and double-strand breaks represent different trapped forms of topoisomerase II–DNA complexes.

While this work was in progress, several reports have appeared from the laboratory of Dr. Leroy Liu which show directly the effects of DNA intercalating agents on purified calf thymus topoisomerase II (Nelson et al., 1984; Tewey et al., 1984). This work has shown the trapping of DNA strand breaks at specific sites in a DNA sequence.

Experimental Procedures

Materials

2-Methyl-9-hydroxyellipticinium acetate was a gift from Dr. J. B. LePecq, Institut Gustave-Roussy, Villejuif, France. Coumermycin and novobiocin were purchased from Sigma Chemical Co. (St. Louis, MO). All other drugs were obtained from the Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD. *m*-AMSA was dissolved in dimethyl sulfoxide at 10 mM. All other drugs were dissolved in nucleus buffer [0.15 M NaCl, 5 mM $MgCl_2$, 1 mM KH_2PO_4, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.1 mM dithiothreitol, and 10% glycerol, pH 6.5].

SV40 DNA (unlabeled and ^3H labeled) and λ 5′-exonuclease were purchased from Bethesda Research Laboratories Inc., Gaithersburg, MD. 3′-Nucleotidase was purchased from Sigma Chemical Co., St. Louis, MO.

Methods

Filter Binding Assay of DNA–Protein Linking. Protein samples were incubated with 10–15 ng of ^3H-SV40 DNA (3000–5000 dpm) for 20 min at 37 °C in 0.2 mL of nucleus buffer. DNA intercalating drugs, when used, were added after DNA and just before addition of the protein sample. Reactions were stopped by addition of 1.0 mL of ice-cold 20 mM disodium ethylenediaminetetraacetate (Na_2EDTA), pH 10, and this solution was then added to 5 mL of the EDTA solution on a poly(vinyl chloride) filter (Millipore type BS, 25-mm diameter, 2-μm pore size). The solution was then allowed to flow out through the filter without applied suction. Filters were then washed with 3 mL of 2 M NaCl, 0.2% Sarkosyl NL30, and 40 mM EDTA, pH 10, and then with 3 mL of 20 mM NaEDTA, pH 10. The effluents and filter washes were collected separately; 3 mL of each was mixed with 10 mL of Aquassure (New England Nuclear Corp., Boston, MA), and radioactivity was determined by scintillation counting. Radioactivity on the filters was determined by heating of the filters in the presence of 1 M HCl, followed by treatment with NaOH, as previously described (Kohn et al., 1981).

Purification of Topoisomerases. L1210 cells were grown in RPMI 1630 medium containing 15% fetal calf serum. Cells were maintained in exponential growth (doubling time 13–15 h) in roller bottles at 37 °C. Between 4 and 10^9 and 9×10^9 cells were harvested; 10^9 cells were processed per 50-mL centrifuge tube. The cells were sedimented, washed twice with iced nucleus buffer, and suspended in 5 mL of iced buffer. The cell suspension was then mixed with 45 mL of nucleus buffer containing 0.35% Triton X-100 (New England Nuclear Corp.) and 0.1 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma Chemical Co., St. Louis, MO). This suspension was mixed gently by rotation for 20 min at 4 °C and then centrifuged at 2000 rpm for 7 min. The nuclei were resuspended and washed in Triton-free buffer and were again centrifuged. The nuclear pellet was extracted for 20 min with 1.0 mL of ice-cold nucleus buffer containing 0.35 M NaCl and then centrifuged at 3000 rpm for 7 min. The supernatant was centrifuged again to remove any remaining nuclei. The crude extract was fractionated through a C26/100 Sephacryl S-400 column (Pharmacia Fine Chemicals, Uppsala, Sweden) at a flow rate of 0.3 mL/min. The active fractions were adsorbed to a DNA–cellulose column (0.7 × 10 cm) in nucleus buffer with the NaCl concentration adjusted to 0.15 M. Denatured calf thymus DNA–cellulose was obtained from Enzo Biochemicals Inc., New York, NY. The column was eluted with a linear 0.15–0.35 M NaCl gradient at 0.25 mL/min. Active fractions from the DNA–cellulose column were pooled and layered onto a 15–40% glycerol gradient in nucleus buffer (0.35 M NaCl). The gradient was centrifuged in a Beckman SW40 rotor at 35 000 rpm for 68 h at 4 °C. The gradients were collected from the bottom.

Polyacrylamide–sodium dodecyl sulfate (NaDodSO$_4$) gel electrophoresis was carried out by the method of Laemmli (1970). Resolving gels of 7.5% polyacrylamide were run at 40 mA for 5 h, stained with 0.25% Coomassie Blue and 50% trichloroacetic acid for 30 min, and destained in 25% methanol and 10% acetic acid overnight.

Two-dimensional gel electrophoresis was carried out in 1.2% agarose at 40 V for 16 h. The gel was then rotated 90°, and 1 μM ethidium bromide was added to the electrophoresis buffer [44 mM tris(hydroxymethyl)aminomethane–acetate (Tris–acetate) and 20 mM EDTA, pH 8.6]. Electrophoresis in the second dimension was at 40 V for 6 h. Gels were destained in 1 mM $MgSO_4$ for 15 min and photographed under ultraviolet light.

KDodSO$_4$ Coprecipitation of DNA–Protein Complexes. The precipitation method used was derived from that described by Liu et al. (1983). Reaction mixtures were stopped by the addition of 1.5 volumes of 1% NaDodSO$_4$, 10 mM Tris, 1 mM EDTA, and 10 μg/mL bovine serum albumin, pH 7.6. To this was added 0.33 reaction volume of 2.5 M KCl, 10 mM Tris, and 1 mM EDTA, pH 7.6, and the tubes were placed in ice. The precipitate was washed twice with 0.2 M KCl and then dissolved in nucleus buffer at 37 °C.

Results

Partial Purification of m-AMSA-Dependent DNA-Linking Protein. Previous studies of intact cells and isolated nuclei using the alkaline elution method indicated that the DNA–protein linking activity we were looking for would be stabilized by DNA intercalating agents, such as *m*-AMSA, and that the DNA–protein complexes would remain adsorbed to poly(vinyl chloride) filters in the presence of 2 M NaCl, 0.2% Sarkosyl, and 0.04 M EDTA, pH 10 (Kohn et al., 1981). An activity obeying these criteria was found in a 0.35 M NaCl extract prepared from isolated nuclei essentially as described by Filipski et al. (1983). The extract was fractionated through a Sephacryl S-400 gel filtration column in nucleus buffer (0.15 M NaCl, 5 mM $MgCl_2$, 1 mM KH_2PO_4, 1 mM EGTA, 0.1 mM dithiothreitol, and 10% glycerol, pH 6.5), and the fractions were assayed for DNA–protein linking by means of a filter method based on the retention of radioactive DNA by protein-absorbing filters (see Experimental Procedures) (Figure 1). Gel filtration fractions containing DNA-linking protein were loaded onto a DNA–cellulose column in nucleus buffer. Approximately 60% of the loaded protein did not adsorb to the denatured DNA in the column, and this nonadsorbed protein had no DNA-linking activity. The column was de-

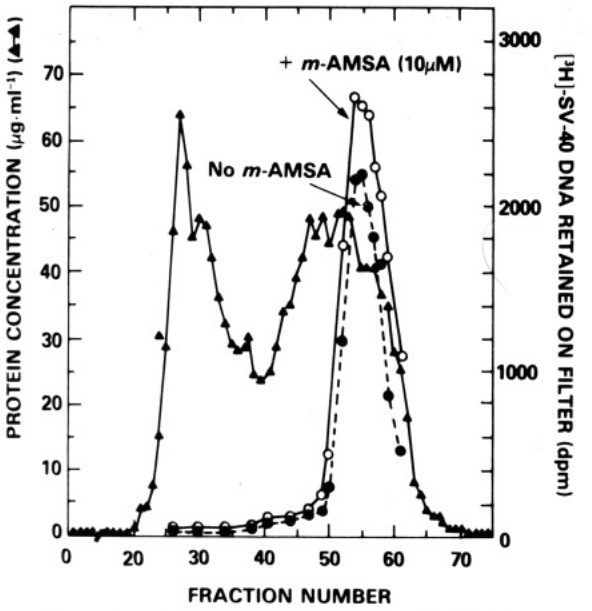

FIGURE 1: Sephacryl S-400 gel filtration of 0.35 M NaCl extract of L1210 cell nuclei. DNA-linking protein in 10 μL of extract was assayed in the presence (○) and absence (●) of 10 μM *m*-AMSA, as described under Experimental Procedures.

Table I: Purification of the *m*-AMSA-Dependent, DNA-Linking Protein[a]

	total protein (mg)	DNA–protein linking act.	
		m-AMSA (10 μM)	no drug
0.35 M NaCl nuclear extract	13.9	0.15 + 0.03	0.06 ± 0.02
Sephacryl S-400 gel filtration	3.8	0.85 ± 0.10	0.36 ± 0.08
DNA–cellulose chromatography	0.7	3.76 ± 0.16	1.69 ± 0.06

[a] DNA–protein linking activity is expressed as nanograms of ^{3}H-SV40 DNA retained on the filter per 100 ng of protein when DNA filter retention is 50%.

veloped by using a linear 150–350 mM NaCl gradient (Figure 2). Approximately one-third of the adsorbed protein was eluted between 180 and 200 mM NaCl and had no DNA-linking activity in our assay. The DNA-linking activity consistently eluted at 290–350 mM NaCl, and the specific activity was constant over the range of this peak (Figure 2). The partial purification of DNA-linking protein and its *m*-AMSA dependence are summarized in Table I. The DNA-linking protein fraction recovered from the DNA–cellulose column represented about 5% of the protein in the 0.35 M NaCl extract of nuclei.

The proteins in the crude extract and partially purified fractions were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 3). A major band of protein at 168 kilodaltons (kDa) was present in the active DNA–cellulose fraction (lane 3). In this experiment, there were also two fainter bands in the neighborhood of 66 kDa and several very faint bands. In other preparations, the 168-kDa band and its faint satellites were the only protein bands visible in the DNA–cellulose fraction, and the bands near 66 kDa were absent. The 168-kDa protein from the DNA–cellulose column was partially depleted when the sample was preincubated with DNA (lane 5) and further depleted by DNA plus *m*-AMSA (lane 6), whereas *m*-AMSA alone (lane 4) produced no depletion. The fainter bands near 66 kDa were not depleted by any of these treatments. A faint band just below the 168-kDa band also exhibited depletion, as did two or three other faint

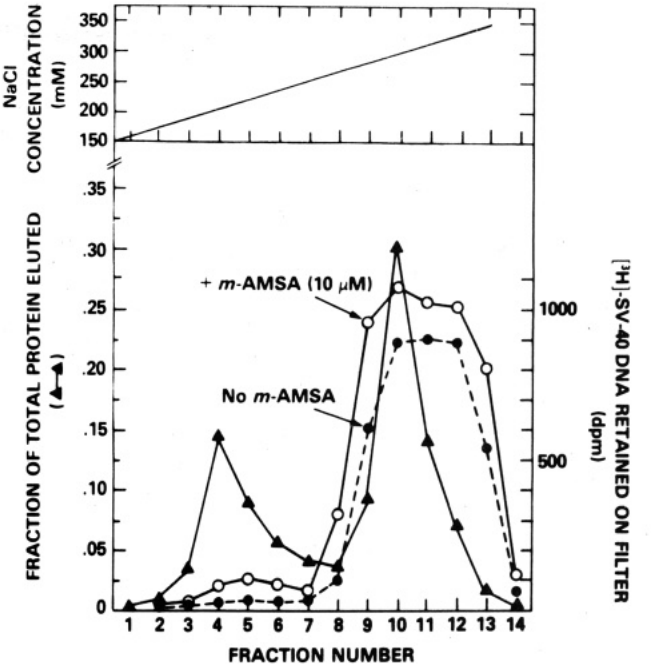

FIGURE 2: Single-strand DNA–cellulose chromatography of *m*-AMSA-dependent DNA-linking protein. Fractions 51–62 from the Sephacryl column (Figure 1) were loaded onto the column and eluted with a gradient of 0.15–0.35 M NaCl in nucleus buffer. DNA-linking protein was assayed by using 150 ng of protein and 9.3 ng of ^{3}H-SV40 DNA in the presence (○) or absence (●) of 10 μM *m*-AMSA.

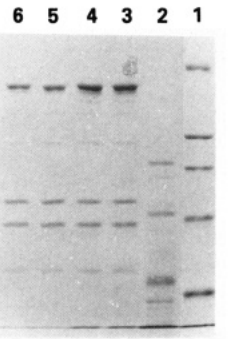

FIGURE 3: $NaDodSO_4$–polyacrylamide (7.5%) gel electrophoresis of partially purified extract. Proteins were stained with Coomassie blue. Lane 1, protein markers; lane 2, material from Sephacryl column that did not adsorb to DNA–cellulose (6 μg of protein); lane 3, DNA-linking (filter assay) fraction recovered from DNA–cellulose column (4 μg of protein); lane 4, same as lane 3 but incubated with 10 μM *m*-AMSA (20 min at 37 °C); lane 5, same as lane 3 but incubated with 25 μg of calf thymus DNA; lane 6, same as lane 3 but incubated with both *m*-AMSA and calf thymus DNA.

bands in the range of 100–150 kDa. The depletion of the 168-kDa protein was probably due to covalent binding of the protein to DNA, because the depletion was seen despite treatment of the protein–DNA mixture with $NaDodSO_4$ and 2-mercaptoethanol at 100 °C (as part of the standard procedure of sample preparation for $NaDodSO_4$–polyacrylamide electrophoresis). The very faint bands in the 100–150-kDa range were probably breakdown products of the 168-kDa protein, because their quantity increased upon storage at 4 °C for 1–2 weeks. Sander & Hsieh (1983) observed a similar family of topoisomerase II bands (from *Drosophila*) and also concluded that they represent proteolytic products of a 170-kDa parent molecule.

Further work disclosed that the material isolated following DNA–cellulose chromatography contained both topoisomerase I and topoisomerase II activities. These two activities were separated by centrifugation through a glycerol gradient.

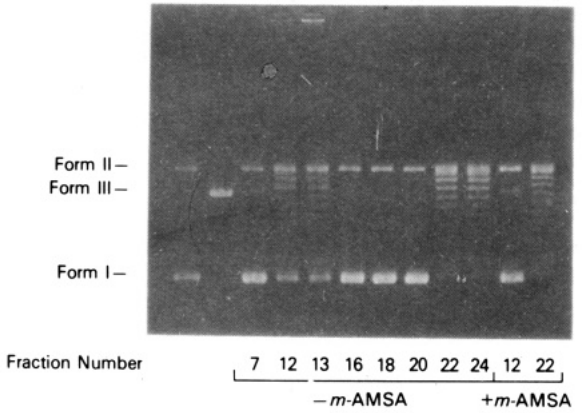

FIGURE 4: Separation of topoisomerases I and II by glycerol gradient centrifugation. 0.5 mL of the DNA–cellulose-purified material was centrifuged through a 10-mL 15–40% glycerol gradient in nucleus buffer containing 0.35 M NaCl at 35 000 rpm for 68 h at 4 °C. The fractions (20-μL aliquots) were reacted with 0.7 μg of SV40 DNA in nucleus buffer containing 0.5 mM ATP and 50 μg/mL bovine serum albumin. Reactions were stopped with 0.4% $NaDodSO_4$ and 0.2 μg/mL proteinase K. Samples were electrophoresed in 1% agarose at 35 V for 16 h. The gels were stained with ethidium.

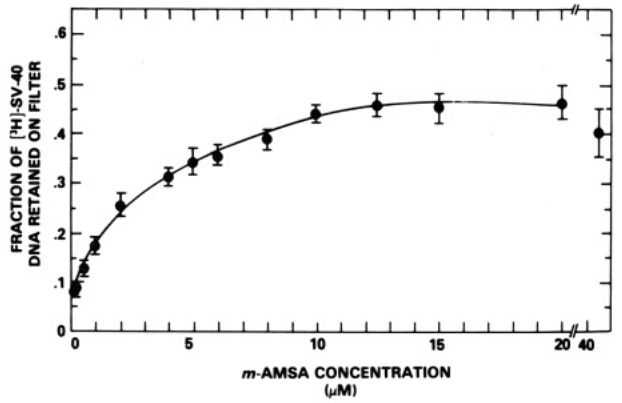

FIGURE 5: Dependence of DNA-linking activity on *m*-AMSA concentration. Reaction mixtures contained 0.75 ng of DNA–cellulose-purified protein, 9.5 ng of ^{3}H-SV40 DNA, and *m*-AMSA in 0.2 mL of nucleus buffer; incubation was for 20 min at 37 °C. Filter retention was assayed as described under Experimental Procedures. The retention for DNA plus 20 μM *m*-AMSA alone was <0.01. Error bars represent the standard deviations of triplicate determinations performed concurrently on the same protein preparation.

Figure 4 shows analyses of representative fractions from such a gradient. Topoisomerase activity, tested in the presence of ATP (0.5 mM), appeared in fractions 11–14 and 21–24. The topoisomerase activity in fractions 11–14 was ATP dependent and produced a band near the top of the gel which may represent catenanes. This activity also produced DNA double-strand cleavage and strong protein–DNA binding (in filter binding assays) that were enhanced in the presence of *m*-AMSA. In addition, *m*-AMSA inhibited the topoisomerization reactions (Figure 4). The activity in fractions 21–24 did not require ATP for DNA relaxation and therefore is probably a topoisomerase I. This topoisomerase I did not produce DNA binding in our filter assay, either in the presence or in the absence of *m*-AMSA, and its ability to relax supercoils was unaffected by *m*-AMSA.

Characteristics of DNA–Protein Linking Activity. The enhancement of DNA–protein linking by *m*-AMSA is an essential feature of the activity of interest. Therefore, we were puzzled by the small magnitude of the enhancement by *m*-AMSA in the assays of the peaks in the gel filtration and DNA–cellulose fractionations (Figures 1 and 2). We noted, however, that the degree of enhancement was larger along the ascending and descending flanks of the peaks where the quantity of active protein used in the assays was low. In assays using relatively low concentrations of active protein, there was considerable dependence on *m*-AMSA (Figure 5). The extent of filter binding activity in the absence of *m*-AMSA varied among different enzyme preparations. In the absence of *m*-AMSA, the dependence of DNA linking on the concentration of active protein was S shaped, such that DNA linking was prominent at high protein concentration but slight at low concentration (Figure 6). In the presence of 10 μM *m*-AMSA, DNA linking rose steeply as protein was added without an initial lag. Thus, the dependence of DNA linking on *m*-AMSA (ratio in the presence and absence of *m*-AMSA) was much greater at low than at high protein concentrations.

Qualitatively similar results were obtained with glycerol gradient purified topoisomerase II, although the S-shaped character of the binding curve in the absence of *m*-AMSA was not as prominent as it was in the case of the DNA–cellulose fraction (E. Uhlenhopp, unpublished results).

Alkaline elution studies of intact cells treated with *m*-AMSA had indicated that the presumed covalent DNA–protein complex is stable at pH 12 and adsorbs to poly(vinyl chloride) filters at this pH (Zwelling et al., 1981). DNA–protein complexes formed from SV40 DNA and purified protein extract, adsorbed on poly(vinyl chloride) filters, were therefore exposed to a pH 12 solution of tetrapropylammonium hydroxide–EDTA, similar to that which is used in alkaline elution (Figure 7). It was found that approximately 50% of the SV40 DNA remained adsorbed to the filter after wash with the pH 12 solution. It may be that in this experiment only one strand of each SV40 DNA molecule became protein linked.

Several additional features of the DNA–protein linking reaction were consistent with the characteristics of the intercalator-dependent formation of protein-associated DNA strand breaks in intact cells and isolated nuclei (Zwelling et al., 1981; Pommier et al., 1984a): (1) the DNA–protein linking activity occurred at 37 and 22 °C but not at 4 °C (Table II); (2) the activity was inhibited by 1 mM EDTA; (3) the activity was inhibited by 0.3 M NaCl; and (4) the activity was stimulated by certain other DNA intercalators, including

Table II: Effects of Temperature and EDTA and Salt Concentrations on DNA-Linking Activity[a]

condition	−*m*-AMSA	+*m*-AMSA
37 °C	0.21 ± 0.02	0.64 ± 0.04
22 °C	0.22 ± 0.04	0.60 ± 0.05
4 °C	0.04 ± 0.02	0.05 ± 0.01
1 mM EDTA	0.07 ± 0.03	0.11 ± 0.02
200 mM NaCl	0.18 ± 0.04	0.57 ± 0.06
300 mM NaCl	0.03 ± 0.01	0.02 ± 0.01

[a] Reaction mixtures contained 100 ng of DNA–cellulose-purified protein and 11 ng of ^{3}H-SV40 DNA, with or without 10 μM *m*-AMSA, in 0.2 mL of nucleus buffer; incubation was for 20 min at 37 °C, unless otherwise indicated. Values are the fraction of DNA retained on the filter (see Experimental Procedures); mean ± SD of three experiments.

Table III: Effects of Various DNA Intercalating Agents on DNA Binding Activity of DNA–Cellulose-Purified Protein[a]

drug	concn	filter retention
none		0.04 ± 0.01
m-AMSA	10 μM	0.42 ± 0.04
ellipticine	20 μM	0.31 ± 0.06
2-methyl-9-hydroxyellipticinium	80 μM	0.07 ± 0.01
5-iminodaunorubicin	10 μM	0.12 ± 0.03
adriamycin	0.10 μM	0.13 ± 0.04
bleomycin	200 μg mL^{-1}	0.03 ± 0.01

[a] Experimental procedure similar to that described in Table II.

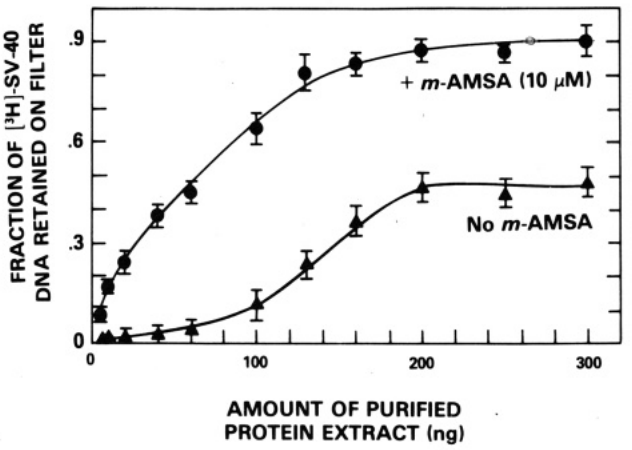

FIGURE 6: Dependence of DNA linking on the concentration of partially purified protein in the presence and absence of *m*-AMSA. Reaction mixtures contained the indicated amounts of DNA–cellulose-purified protein, 12 ng of ^{3}H-SV40 DNA, and 10 μM *m*-AMSA (●), or no *m*-AMSA (▲), in 0.2 mL of nucleus buffer; incubation was for 20 min at 37 °C. Error bars indicate standard deviations of triplicate determinations. (In this experiment, a limited fraction of the DNA became filter-bound at high protein concentrations in the absence of *m*-AMSA, but in some experiments, filter binding in the absence of *m*-AMSA rose to 80–90%.)

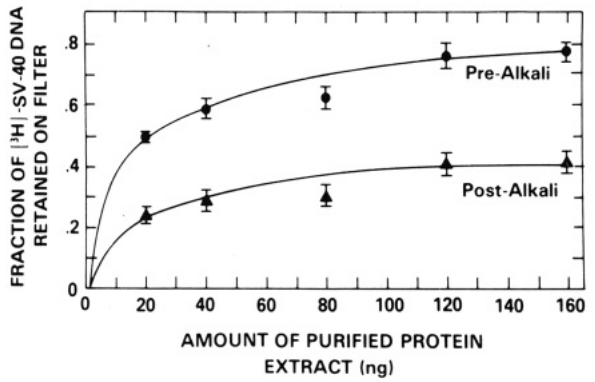

FIGURE 7: Effect of alkali (pH 12.1) on the retention of *m*-AMSA-dependent DNA–protein complexes on poly(vinyl chloride) filters. Reaction mixtures (0.2 mL) contained 11 ng of ^{3}H-SV40 DNA, 10 μM *m*-AMSA, and the indicated amounts of DNA–cellulose-purified protein. The reaction mixtures were incubated for 20 min at 37 °C and were then passed through poly(vinyl chloride) filters which were then washed with 2 M NaCl, 0.2% Sarkosyl, and 0.04 M EDTA, pH 10, as described under Experimental Procedures. Filters were then either processed for scintillation counting or treated further with 20 mL of tetrapropylammonium hydroxide/EDTA, pH 12.1 [the alkaline eluting solution described by Kohn et al. (1981)]. After contact with the filter for 15 min at 22 °C, this solution was allowed to flow out through the filter with no applied suction.

ellipticine, 5-iminodaunorubicin, and adriamycin, but not by bleomycin (Table III). 2-Methyl-9-hydroxyellipticinium (80 μM) had only a slight stimulatory effect; this compound has been found to stimulate protein-associated DNA scission in isolated nuclei at low concentration but to be inhibitory at high concentration (Pommier et al., 1982).

To determine whether protein linking occurred at 3′ or 5′ DNA termini, sensitivity to 3′- and 5′-exonucleases was tested by using ^{3}H-adenovirus 2 DNA. After treatment with purified extract (4 μg of protein) plus *m*-AMSA (10 μM for 20 min at 37 °C), 86% of the adenovirus DNA adsorbed to the filters. The DNA was then made single stranded by exposure to alkali and incubated for 16 h at 37 °C with either 3′- or 5′-exonuclease. The 3′-exonuclease released 98% of the DNA from the filter, whereas the 5′-exonuclease released only 19%. The released DNA fragments were at least 90% acid soluble. (Treatment of control DNA by 3′- and 5′-exonuclease yielded

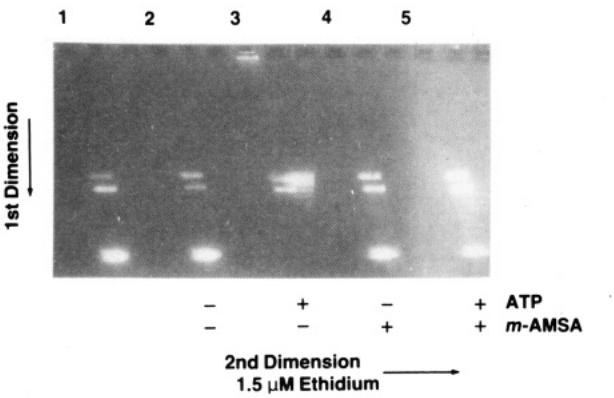

FIGURE 8: Effects of *m*-AMSA on glycerol gradient purified topoisomerase II. Reaction mixtures (40 μL) contained 5 μL of enzyme fraction and 0.8 μg of SV40 DNA. Reactions were for 20 min at 37 °C and were stopped with 0.4% NaDodSO$_4$ and proteinase K. Two-dimensional electrophoresis was as in Figure 9. Lane 1, mixture of native and linearized SV40 DNA; lane 2, DNA incubated with topoisomerase II without added ATP; lane 3, same as lane 2 with 0.2 mM ATP added; lanes 4 and 5, same as lanes 2 and 3, respectively, but with 10 μM *m*-AMSA added.

90% and 83% acid solubility, respectively.) Thus, the 5′, but not 3′, termini were protected from exonuclease digestion, presumably because of protein linking at the 5′ termini.

DNA Strand Scission and Inhibition of Topoisomerization. The effects of *m*-AMSA on the glycerol gradient purified enzyme are illustrated in Figure 8. The effects of *m*-AMSA on the DNA strand scission and topoisomerization activities of the enzyme at different stages of purification were investigated by two-dimensional electrophoresis in 1% agarose gels. The first dimension separated three bands consisting, in the order of highest to lowest electrophoretic mobility, of supercoiled, linear, and a mixture of relaxed and nicked circles. The latter mixture was separated in the second dimension and run with added ethidium which increased the mobility of the closed double circles relative to the nicked circles. In the absence of *m*-AMSA and ATP, only a small amount of the DNA was in the nicked or double-strand-cut states (lane 2), and no covalently closed topoisomers were formed. When ATP was added, all of the supercoiled molecules were converted to topoisomers and to material in a band near the top of the gel which may represent catenanes (sample 3). This is as expected for topoisomerase II. When *m*-AMSA was added without ATP, there were approximately equal increases in nicked and double-strand-cut DNA (sample 4). The addition of ATP plus *m*-AMSA further increased the amounts of DNA trapped in nicked and double-strand-cut forms (sample 5); the nonhydrolyzable ATP analogues 5′-adenylyl imidodiphosphate (AMPPNP) and adenosine 5′-O-(3-thiotriphosphate) (ATPγS) also had this effect (data not shown).

The less purified preparation after the DNA–cellulose step exhibited similar properties except for the presence of topoisomerase I activity which generated topoisomers in the absence of ATP.

Stabilization of Protein-Associated DNA Strand Breaks. To show that the DNA–protein links and DNA strand breaks generated by topoisomerase II in the presence of *m*-AMSA are associated, as they are known to be in the absence of *m*-AMSA (Sander & Hsieh, 1983; Liu et al., 1983), the experiment illustrated in Figure 9 was performed. Reaction mixtures of DNA–cellulose-purified proteins and SV40 DNA were stopped with NaDodSO$_4$, and the protein-linked DNA was then precipitated with KCl. The DNA species in the supernatant and pellet were analyzed by two-dimensional gel electrophoresis, either with or without prior proteinase

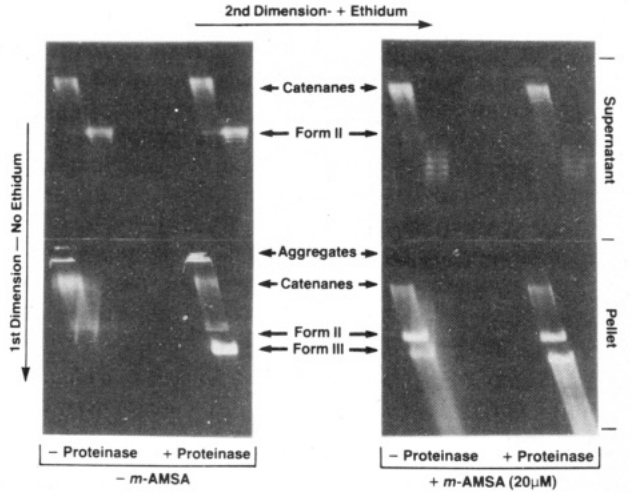

FIGURE 9: Stabilization of protein-associated DNA strand breaks by *m*-AMSA when precipitated by $NaDodSO_4$–KCl. DNA–cellulose-purified protein was reacted with SV40 DNA with and without *m*-AMSA present in nucleus buffer for 20 min at 37 °C. Reactions were stopped by the addition of 3 volumes of 50 mM Tris (pH 7.6), 1% $NaDodSO_4$, 10 μg/mL bovine serum albumin, and 1 mM EDTA. Tubes were iced, and an equal volume of 50 mM Tris (pH 7.6), 2.5 M KCl, and 1 mM EDTA was quickly added and vortex-mixed. After precipitates were allowed to form for 60 min on ice, pellets were collected by centrifugation (12000*g* for 5 min). The pellets were dissolved in 10 mM Tris and 20 mM EDTA (pH 10) at 37 °C. Aliquots of this solution and of the supernatant were incubated with proteinase K for 60 min at 37 °C. Electrophoresis was in 1% agarose downward for 16 h at 35 V and then from left to right for 5 h in the presence of 1 μM ethidium.

treatment to detect protein-linked species. The use of the DNA–cellulose-purified proteins allowed the actions of both topoisomerase I and topoisomerase II to be observed in a minimally processed preparation. Since no ATP was added, only topoisomerase I produced topoisomers. These were found only in the supernatant and were unaffected by *m*-AMSA except in the altered positions of the topoisomers expected due to DNA intercalation by *m*-AMSA during the reaction. There was no nicked or linear DNA in the supernatant. All of the nicked and linear DNA was precipitated, indicating that it was covalently linked to protein.

This experiment was done with a relatively high protein/DNA ratio so that a large fraction of DNA became protein linked even in the absence of *m*-AMSA. The DNA in these protein-linked complexes was mainly recovered as linearized forms, resulting from a double-strand cut. *m*-AMSA had two effects: first, it trapped single-strand-cut as well as double-strand-cut DNA species; second, it produced short DNA fragments resulting from multiple double-strand cuts per DNA molecules (revealed by the downward smear in the lower right corner of Figure 9).

Another observation was that *m*-AMSA appeared to release cut DNA from the protein complex in the absence of proteinase. This must have occurred during the resolubilization of the pellet at 37 °C.

Effect of m-AMSA on the Reversibility of the Complex. The cleavable complex of purified topoisomerase II and DNA has been shown to be reversed when the reaction mixture is cooled to 0 °C (Liu et al., 1983). In intact cells and isolated nuclei, however, the protein-associated DNA strand breaks produced in the presence of *m*-AMSA persisted at 0 °C following drug removal, although they did reverse at 37 °C (Zwelling et al., 1981; Pommier et al., 1982). This difference in cold reversibility was investigated in *m*-AMSA-induced cleavable complexes of purified topoisomerase II and SV40

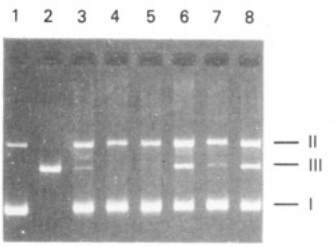

FIGURE 10: Effect of *m*-AMSA on reversibility of the DNA–topoisomerase II complex. SV40 DNA (7 μg) was reacted with glycerol gradient purified topoisomerase II in nucleus buffer containing 50 μg/mL bovine serium albumin (no ATP) for 20 min at 37 °C. The reaction mixture either included *m*-AMSA (10 μM) (lanes 6–8) or did not include *m*-AMSA (lanes 3–5). Aliquots of the reaction mixture were then incubated for 5 min either under the same conditions (lanes 3 and 6), or after addition of NaCl to 0.55 M (lanes 4 and 7), or after transferring the aliquot to a prechilled tube in an ice bath (lanes 5 and 8). The reactions were then stopped by addition of $NaDodSO_4$ to 0.4% and proteinase K to 50 μg/mL. Lane 1, SV40 DNA; lane 2, *Eco*RI digest of SV40 DNA. Samples were electrophoresed in a 1% agarose gel.

Table IV: Effect of *m*-AMSA on Reversibility of the DNA–Topoisomerase II Complex[a]

"reversal" condn	fraction of DNA retained on filter[b]	
	no drug added	*m*-AMSA added
control	0.16 ± 0.03	0.52 ± 0.04
0.55 M NaCl	0.03 ± 0.01	0.37 ± 0.05
4 °C	0.05 ± 0.02	0.49 ± 0.06

[a] Mixtures (0.2 mL) contained 0.27 μg of ^{3}H-SV40 DNA, 3.0 μg of DNA–cellulose-purified protein, and, when indicated, 10 μM *m*-AMSA. Reactions were for 20 min at 37 °C. Aliquots of the reaction mixture were then incubated for 5 min either under the same conditions (control), or after addition of NaCl to 0.55 M, or after transferring the aliquot to a prechilled tube in an ice bath. Covalent DNA–protein complexes were assayed by filter binding, as described under Experimental Procedures. [b] Mean ± SD of three assays.

DNA (Figure 10). Cleavable complexes formed in the absence of *m*-AMSA reversed upon cooling, as previously reported. In the presence of *m*-AMSA, however, cooling did not reduce the cleavable complexes, when assayed either by DNA–protein binding (filter adsorption assay, Table IV) or by DNA strand scission (Figure 10). The reversal of cleavable complexes by 0.5 M NaCl (Liu et al., 1983) was inhibited by *m*-AMSA only to a small extent (Table IV, Figure 10).

Discussion

This work aimed to isolate and identify the source of an effect of DNA intercalating agents that had been observed first in intact cells (Ross et al., 1979; Zwelling et al., 1981) and then in isolated nuclei (Filipski & Kohn, 1982; Pommier et al., 1982). The effect was characterized by the formation of DNA strand breaks and DNA–protein cross-links at apparently equal frequencies and localized with respect to each other (Pommier et al., 1984b). In isolating this activity, we used a minumum number of simple separation steps in an attempt to avoid altering the activity during the isolation process. The presence of an activity with the requisite properties in a 0.35 M NaCl extract of isolated nuclei and its fractionation by gel filtration were previously reported (Filipski et al., 1983).

The progress of the current work was speeded by the development of a rapid filter assay for the intercalator-dependent DNA–protein binding (see Experimental Procedures). An interesting feature of this method is that the protein–DNA complex of interest remains adsorbed to the filters despite a wash with 2 M NaCl, 0.2% Sarkosyl, and 0.02 M EDTA, pH

10. This is the composition of the lysis solution used in the studies of intact cells and isolated nuclei by the alkaline elution technique (Kohn et al., 1981).

Only two additional fractionation steps were used after gel filtration. The first was a DNA–cellulose column separation of a class of relatively tight DNA binding proteins. (The very tight DNA binding proteins such as histones were not released from the isolated nuclei by the initial 0.35 M NaCl extraction). In the current procedure, both topoisomerase I and topoisomerase II activities were recovered in this step. The studies of the effects of the intercalator *m*-AMSA were designed so as to distinguish effects on either topoisomerase. The two topoisomerase activities were then separated by glycerol gradient centrifugation, and the effects of *m*-AMSA were examined.

The effects of *m*-AMSA on the activities of the topoisomerase II fraction could account for the production of protein-associated DNA strand breaks in intact cells. *m*-AMSA stimulated the covalent binding to DNA of protein from the semipurified topoisomerase preparation as well as from the glycerol gradient purified topoisomerase II but not from the topoisomerase I component. The binding was to the 5′ DNA termini, as expected for topoisomerase II (Liu et al., 1983), and was associated with the production of both single- and double-strand breaks, as observed in intact cells. This mechanism thus accounts for both the single- and double-strand breaks produced by *m*-AMSA in intact cells. The *m*-AMSA-induced trapping of cleavable DNA–topoisomerase II complexes occurred in the absence of added ATP and was reversible at 22 or 37 °C, consistent with previous observations in isolated nuclei (Pommier et al., 1982, 1984a). The *m*-AMSA-induced complexes, however, were not reversed at 4 °C, consistent with the previously observed inhibition of the reversal of protein-associated DNA strand breaks in intact cells at this temperature (Zwelling et al., 1981). This contrasts with the reversal by cooling in ice of cleavable topoisomerase II–DNA complexes formed in the absence of intercalator (Liu et al., 1983), showing that *m*-AMSA alters this property similarly in intact cells and purified topoisomerase II.

The formation of covalent DNA–topoisomerase II complexes was stimulated to various degrees by different intercalators, and the relative magnitudes for the different compounds were consistent with the pattern that has been found in intact cells (Zwelling et al., 1982).

In the presence of ATP, topoisomerase II, when acting on covalently closed DNA circles, normally produces topoisomers and catenanes. When *m*-AMSA was present in addition to ATP, however, topoisomerization and catenane production were inhibited. ATP enhanced the *m*-AMSA-induced trapping of complexes in which the DNA has single- or double-strand cuts. Nonhydrolyzable ATP analogues also tended to trap some of the DNA as single- and double-strand-cut forms and enhanced the effect of *m*-AMSA. Similar results had been obtained in isolated nuclei (Pommier et al., 1984a).

It has not been possible to observe topoisomerization by topoisomerase II in intact cells directly. An effect of *m*-AMSA on DNA linking number in intact cells has been interpreted, however, as possibly resulting from an inhibition of an active DNA unwiding process (Pommier et al., 1984b,c). Purified eukaryotic topoisomerase II has not been demonstrated to actively unwind DNA helices, but the possibility of such an action in intact cells is not excluded.

The formation of covalent protein–DNA complexes in our topoisomerase II extracts occurred even in the absence of intercalator when relatively high concentrations of extracts were used. Although alternative explanations are possible, an interesting possibility is that topoisomerase II exists in cells in two states, one of which binds to DNA but does not pass DNA helices whereas the other produces topoisomerization reactions but does not normally form stable DNA complexes. The observed S-shaped dependence of DNA–protein binding on the concentration of extract may reflect the presence of a dissociable component that converts topoisomerase II from one state to another. A recently reported property of epidermal growth factor receptor, the ATP-stimulated nicking of supercoiled DNA (Mroczkowski et al., 1984), could represent topoisomerase II in the DNA binding state.

Our results support the view that the stimulation of protein-associated DNA strand breaks by treatment of cells with *m*-AMSA can serve as a functional measure of topoisomerase II in cells. We have previously observed changes in this measure in cells after prolonged partial inhibition of DNA synthesis with arabinosylcytosine or hydroxyurea (Minford et al., 1984b), and studies in progress show that marked changes in this measure occur during the cell cycle.

While this work was in progress [a preliminary report has appeared (Minford et al., 1984a)], studies in the laboratory of Leroy Liu have independently demonstrated the stimulation by *m*-AMSA of the formation of complexes between purified mammalian topoisomerase II and DNA in which DNA single- or double-strand breaks are linked at their 5′- termini to the enzyme (Nelson et al., 1984; Tewey et al., 1984).

In regard to the mechanism of the trapping of covalent topoisomerase II–DNA complexes by DNA intercalators, we find that ATP and nonhydrolyzable ATP analogues enhance trapping. Thus, occupancy of the ATP site on the enzyme appears to enhance trapping, while ATP hydrolysis, i.e., energy input into the system, is not required. The small (and variable) extent of trapping that we see in the absence of added ATP could be due to the carryover of a small amount of ATP which may remain bound to the enzyme during isolation. It will in the future be necessary to take into account the fact that topoisomerase II is a dimer and that therefore at least two ATP sites may have to be occupied to make possible the conformational change that must take place during strand passage.

It seems plausible that the conformational changes associated with strand passage would require several conditions: (1) a change in the ATP sites which can occur only if ATP (or an analogue) is present at the sites; (2) the opening of one or both DNA strands with covalent linkage of the 5′ DNA termini to tyrosine residues on the enzyme; and (3) the presence of a helical DNA segment that is to be the passing strand. We propose that these are coordinated events and that none will occur unless all can take place. This picture leads to an attractive model of how certain DNA intercalators trap covalent complexes. We propose that trapping occurs when a suitable intercalator is bound to the *passing strand* in the region that enters a cleft in the enzyme. If the intercalator has an appropriate structure, it stabilizes a complex which is an intermediate in the strand-passing process. Either one or both DNA strands would be open, depending on the exact structure of the intermediate complex that is stabilized. This view would presume that intercalators that do not stabilize covalent complexes do not allow good steric fit of the intercalator-bearing passing strand in the enzyme cleft.

Registry No. *m*-AMSA, 51264-14-3; DNA topoisomerase, 80449-01-0.

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Escherichia coli Tyrosyl- and Methionyl-tRNA Synthetases Display Sequence Similarity at the Binding Site for the 3′-End of tRNA†

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ABSTRACT: Covalent modification of *Escherichia coli* tyrosyl-tRNA synthetase (TyrRS) by the 2′,3′-dialdehyde derivative of $tRNA^{Tyr}$ ($tRNA_{ox}$) resulted in a time-dependent inactivation of both ATP–PP_i exchange and tRNA aminoacylation activities of the enzyme. In parallel with the inactivation, covalent incorporation of approximately 1 mol of [^{14}C]$tRNA_{ox}^{Tyr}$/mol of the dimeric synthetase occurred. Intact $tRNA^{Tyr}$ protected the enzyme against inactivation by the tRNA dialdehyde. Treatment of the TyrRS–[^{14}C]$tRNA^{Tyr}$ covalent complex with α-chymotrypsin produced two labeled peptides (A and B) that were isolated and identified by sequence analysis. Peptides A and B are adjacent and together span residues 227–244 in the primary structure of the enzyme. The three lysine residues in this sequence (lysines-229, -234, and -237) are labeled in a mutually exclusive fashion, with lysine-234 being the most reactive. By analogy with the known three-dimensional structure of the homologous tyrosyl-tRNA synthetase from *Bacillus stearothermophilus*, these lysines should be part of the C-terminal domain which is presumed to bind the cognate tRNA. Interestingly, the labeled TyrRS structure showed significant similarities to the structure around the lysine residue of *E. coli* methionyl-tRNA synthetase which is the most reactive toward $tRNA_f^{Met}$(ox) (lysine-335) [Hountondji, C., Blanquet, S., & Lederer, F. (1985) *Biochemistry 24*, 1175-1180].

Bacterial tyrosyl-tRNA synthetase (TyrRS)[1] is the object of many studies aimed at probing structure–activity relationships. The crystallographic structure of the *Bacillus stearothermophilus* enzyme is presently solved at 3-Å resolution (Bhat et al., 1982). It indicates two domains, one of which, the C-terminal domain, is thought to carry major determinants in tRNA binding. tRNA contacts with tyrosyl-tRNA synthetase have been studied by differential labeling of the lysine residues involved in complex formation (Bosshard et al., 1978). These contact regions include three lysines situated in the C-terminal half of the polypeptide chain of the enzyme (Winter et al., 1983). More recently, the portion of the cloned gene corresponding to the C-terminal region of the enzyme was deleted. The truncated tyrosyl-tRNA synthetase,

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[1] Abbreviations: TyrRS, tyrosyl-tRNA synthetase; MetRS, methionyl-tRNA synthetase; IleRS, isoleucyl-tRNA synthetase; TrpRS, tryptophanyl-tRNA synthetase; $tRNA_{ox}$ or tRNA dialdehyde, the dialdehyde formed by periodate oxidation of the 3′-ribose of tRNA; $tRNA_{ox-red}$, $tRNA_{ox}$ reduced with sodium borohydride; $adenosine_{ox-red}$, the adenosine derivative released from the 3′-end of $tRNA_{ox-red}$ by ribonuclease A; EDTA, ethylenediaminetetraacetic acid.

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