The TyrR Protein of *Escherichia coli*, Analysis by Limited Proteolysis of Domain Structure and Ligand-Mediated Conformational Changes*

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(Received for publication, August 17, 1992)

The TyrR protein of *Escherichia coli* K12 is a homodimer containing 513 amino acids/subunit. This protein is important in the transcriptional regulation of several genes whose protein products catalyze steps in aromatic amino acid biosynthesis or transport. Methods were developed for efficiently purifying the TyrR protein in apparent homogeneity. We analyzed the pattern of cleavage of the TyrR protein by trypsin, either in the absence of ligands or in the presence of saturating levels of L-tyrosine, ATP, or poly(dIl-dC). At low (1:200 ratio by weight) trypsin levels, in the absence of ligands, two major digestion products accumulated. These were polypeptides of 22 and 31 kDa, shown to contain amino acid residues 1-190 and 191-467, respectively. The pattern of trypsin cleavage was unaffected by tyrosine. In the presence of ATP, an intermediate species of 53 kDa, probably containing amino acid residues 1-467, was observed. The kinetics of appearance of the 53-kDa species were consistent with a role for ATP in accelerating the hydrolysis of the R467-F468 peptide bond. The 53-kDa polypeptide underwent further tryptic hydrolysis to yield fragments of 22 and 31 kDa. When both tyrosine and ATP were present, the rate of formation of the 22- and 31-kDa fragments was more rapid than in the absence of these ligands. It appears that when both ligands are bound, the rates of hydrolysis of peptide bonds R190-Q191 and R467-F468 are both enhanced. Additional limited proteolysis experiments suggested that polypeptide segment 191-467 contains ATP binding site(s), and that the rate of cleavage of peptide bonds R190-Q191 and R467-F468 is altered when the TyrR protein interacts with poly(dIl-dC), an analog of target DNA. Our results reveal the presence of two major structural domains within the TyrR protein. The first domain (amino acid residues 1-190) is extremely resistant to hydrolysis by trypsin. The second domain (residues 191-467), which is likely to contain ATP-binding site(s), is homologous to several other transcriptional activators specific for promoters responsive to the σ54 form of RNA polymerase. The remainder of the TyrR protein (residues 468-513) contains the operator recognition elements, probably arranged in the form of a helix-turn-helix motif. This polypeptide segment was not detected as a discrete tryptic hydrolysis product.

The tyrosine repressor (TyrR) protein of *Escherichia coli*,

*This work was supported by United States Public Health Service Grant GM22131. This is Journal Paper 13513 from the Purdue University Agricultural Experiment Station. 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 1794 solely to indicate this fact.

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**Vol. 288, No. 7, Issue of March 5, pp. 5040-5047, 1993**

**Printed in U.S.A.**
with ACE6 at a multiplicity of 5 phage/cell. After 3 h at 37 °C, the culture was grown at 37 °C on a rotary shaker to an OD 600 of about 0.3. The culture was then grown at 37 °C for another hour, and MgSO4 was added to a final concentration of 10 mM. The cells were then infected with the desired concentration of phage ACE6 (10¹⁵/ml) (Rosenberg et al., 1979) containing 0.5% maltose, in a 2-liter flask. The mixture was incubated on a rotary shaker for 6-8 h at 37 °C, at which time lysis occurred. Chloroform (1 ml) was then added to kill any surviving cells and to lyse phage-infected cells. The resulting suspension was centrifuged at 15,000 rpm (Beckman, J21C) for 30 min. The pellet was discarded, and the supernatant material was treated with streptomycin sulfate (10% solution in Buffer A), which was added slowly with stirring to a final concentration of 1%. The resulting suspension was centrifuged at 15,000 rpm (Beckman, J21C) for 30 min. The tube was then spun at 15,000 rpm (Beckman, J21C) for 30 min. In each case, almost all of the TyR protein remained in the supernatant fraction. The relative purity of the TyR protein eluted at different concentrations of NaCl was similar in each case, although the use of 0.5 M NaCl gave the highest yields. At this stage, the TyR protein constituted 50-60% of the total protein.

**Experimental Procedures**

**Materials**

Trypsin and soybean trypsin inhibitor were purchased from Sigma. Polyclonal antibodies were purchased from Boehringer Mannheim. Phosphocellulose P11 was obtained from Whatman. Hydroxylapatite (Bio-Gel HTP) was obtained from BioRad. Chromatographic columns and accessories were purchased from Pharmacia LKB Biotechnology Inc.

**Buffers**

Buffer A was 10 mM K2HPO4/KH2PO4 (pH 6.6) with 100 mM NaCl, 1 mM EDTA, 0.01% NaN3, 7 mM 8-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride.

Buffer B was 50 mM K2HPO4/KH2PO4 (pH 7.5) with 1 mM EDTA, 0.01% NaN3, 7 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride.

**Large Scale Preparation of ACE6**

*E. coli* strain ED8739 (Rosenberg et al., 1987) was grown overnight at 37 °C in 5 ml of TM medium (Gottesman and Yarmolinsky, 1968). 1 ml of cells was added to 10 μl of phage ACE6 (10¹⁵/ml) (Rosenberg et al., 1987). After about 20 min at 37 °C, the phage-cell mixture was transferred into 500 ml of NZC medium (Williams and Blattner, 1979) containing 0.5% maltose, in a 2-liter flask. The mixture was incubated on a rotary shaker for 6-8 h at 37 °C, at which time lysis had occurred. Chloroform (1 ml) was then added to kill any surviving cells and to lyse phage-infected cells. The mixture was centrifuged at 8000 rpm (Sorvall RC2B, GSA rotor) for 20 min at 4 °C. The supernatant, consisting of a high titer phage lysate, was stored at 0-5 °C prior to use. This procedure reproducibly yielded lysates at a titer of 2-3 × 10¹⁰/ml.

**Purification of the TyR Protein**

The procedure for the purification of the TyR protein reported here contains some improvements over the previously described method (Argyropoulos, 1989). To overexpress the TyR protein, strain BL21 (Rosenberg et al., 1987) carrying the tyrr+ construct pJCl00 (Somerville et al., 1991) was first grown overnight at 37 °C in 10 ml of LB medium (Lennox, 1955) supplemented with ampicillin (100 mg/ml) and maltose (0.2%). The overnight culture was then inoculated into 1 liter of the same medium contained in a 4-liter flask. The culture was grown at 37 °C on a rotary shaker to an OD 600 of about 0.3. Glucose was then added to a final concentration of 4 mg/ml. The culture was then grown at 37 °C for another hour, and MgSO4 was added to a final concentration of 10 mM. The cells were then infected with ACE6 at a multiplicity of 5 phage/cell. After 3 h at 37 °C, the cells were harvested by centrifugation at 4 °C, 8000 rpm (Sorvall RC2B, GSA rotor) for 20 min. The TyR protein in these cells comprised 20-30% of the total protein. Protein purification, described below, was performed at 4 °C.

**Step 1**—The cells harvested from a 1-liter culture (3 g of paste) were resuspended in 30 ml of Buffer A. Cells were broken in a French press (three passages at 20,000 psi), then spun at 15,000 rpm (Beckman, J21C) for 30 min. The pellet was discarded, and the supernatant material was treated with streptomycin sulfate (10% solution in Buffer A), which was added slowly with stirring to a final concentration of 1%. The resulting suspension was centrifuged at 15,000 rpm (Beckman, J21C) for 30 min. The TyR protein was then resuspended with the sedimented material. The pellet was then resuspended in several different concentrations of buffer (Buffer A) salt solution, i.e. 0.1, 0.2, 0.3, 0.4, and 0.5 M NaCl. The tubes were rotated slowly for 1 h and then spun at 15,000 rpm (Beckman, J21C) for 30 min. In each case, almost all of the TyR protein remained in the supernatant fraction. The relative purity of the TyR protein eluted at different concentrations of NaCl was similar in each case, although the use of 0.5 M NaCl gave the highest yields. At this stage, the TyR protein constituted 50-60% of the total protein.

**Step 2**—Solid ammonium sulfate was added slowly with stirring to the NaCl eluate to 80% saturation. This treatment precipitated the TyR protein and eliminated some of the unwanted proteins. After dialysis against Buffer A, the sample was loaded onto an 80-ml phosphocellulose P11 column. The column was developed by an NaCl gradient, rising from 0.1 to 1 M in 400 ml. The TyR protein emerged between 375-450 mM NaCl. At this stage, the TyR protein was 90% pure. A second identical phosphocellulose P11 column step further improved the purity of this protein.

**Step 3**—The pooled TyR protein fractions from the phosphocellulose P11 column were concentrated by precipitation with ammonium sulfate (80-85% saturation), resuspended in 10 ml of Buffer B, and dialyzed overnight against 2 liters of Buffer B with three changes. The dialyzed material was then passed in 2-ml aliquots through a 6-ml hydroxylapatite column previously equilibrated with Buffer B. The TyR protein in Buffer B passed directly through the hydroxyapatite column, whereas virtually all of the proteins that had coeluted with the TyR protein from the hydroxylapatite column were retained. At this point, the TyR protein was over 95% pure, as determined by SDS-PAGE (Figs. 2-4; zero time lanes). From 1 liter of paste (about 3 g), 40 mg of homogeneous TyR protein could be reproducibly purified using the above protocol.

**Trypsin Digestion**

Preparations of Trypsin and Trypsin Inhibitor—Trypsin was dissolved in 0.1 nM HCl at a concentration of 1 mg/ml. The stock trypsin solution (100-μl aliquots) was stored at -20 °C. The solution was thawed in an ice bath. Each thawed trypsin stock was used only once. Trypsin inhibitor was dissolved in 20 mM Tris-chloride buffer (pH 7.5) at a concentration of 1 mg/ml and stored at -20 °C.

**Trypsin Digestion**—All digestion experiments were carried out at room temperature (25 °C). Incubation mixtures contained (final volume, 200 μl) 20 mM Tris-chloride (pH 7.5), 1 mM EDTA, 0.5 mM β-mercaptoethanol, and 100 μg TyR protein. Trypsin (either 0.5 or 0.9 μg) was then added to initiate digestion. Tyrosine (500 μM), ATP (200 μM), and polyclonal antibodies to trypsin were added as appropriate. The digestion was terminated at 10 min by boiling the reaction mixture.

**Characterization of Tryptic Fragments**

**Protein Sequencing**—To prepare polypeptides for protein sequencing, a polyvinylidene difluoride-type support, ProBlott (Applied Biosystems) was used with minor modifications, according to the protocols recommended by the supplier. Briefly, SDS-PAGE was conducted according to our routine method (see below) except for the following. 1) A 2-h period of pre-electrophoresis was employed at 3
mM constant current and glutathione (50 μM) was present in the cathode buffer. 2) During electrophoresis, fresh cathode buffer with 0.1 mM sodium thioglycolate was used. After electrophoresis, the poly peptide bands on the gel were transferred electrophoretically to ProBlott membrane in 10 mM CAPS buffer with 10% methanol. The membranes were briefly stained with Coomassie Blue R-200 (0.1%), acetic acid (1%), and methanol (40%). Stained bands were excised and subjected to Edman degradation analysis. Six reaction cycles were performed.

**Purification of the 31-kDa Tryptic Fragment**—Tryptsin digestion of the TyrR protein was carried out as described above. Low levels of trypsin (1:200) were used. Both ATP (200 μM) and tyrosine (500 μM) were added to protect the 31-kDa fragment from further digestion. After 16 min of incubation, the reaction was stopped with trypsin inhibitor as described above. Tryptic fragments were separated on a 10% native polyacrylamide gel. The native gel and running conditions were identical with those employed in SDS-PAGE, except that no SDS was added to either the gel or the electrode buffers. The gel was stained with Coomassie Blue R-200 (0.1%) for 10 min and destained briefly with distilled H2O until the 31-kDa fragment became visible. The fragment was cut out of the gel and eluted using an S&S ELUTRAP apparatus (Schleicher and Schuell), following the supplier’s instructions. The eluted 31-kDa polypeptide was dialyzed overnight at 4 °C against Buffer B. Frozen samples (−20 °C) were used for molecular mass determination (see below).

**Analytical Methods**

Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as standard. Reagents were purchased from Bio-Rad.

SDS-PAGE was carried out in a Mini Protean II cell (Bio-Rad). A tricine buffer system (Schägger and Von Jagow, 1987) was used at a gel concentration of 10%. 2 × loading buffer was used to prepare the samples.

Poly peptide molecular weights were determined by electrospray mass spectrometry by Dr. K. M. Swiderek at the Beckman Research Institute of the City of Hope Medical Center, Duarte, California.

**RESULTS**

**Limited Tryptic Digestion of the TyrR Protein**

**Effects of Ligands**—Limited proteolysis has been extensively used to investigate the structures of multidomain proteins (Rossmann and Argos, 1981). The availability in quantity of highly purified TyrR protein (see "Experimental Procedures") made it feasible to utilize trypsin digestion to study the domain structure of the TyrR protein. There are over 50 possible sites of tryptic cleavage in the TyrR protein (Cornish et al., 1986; Fig. 1). Selective hydrolysis of a subset of these peptide bonds would constitute evidence for their exposure to solvent. This feature of interdomain poly peptide segments is commonly observed (Rossmann and Argos, 1981). Two levels of trypsin were employed. Low levels (trypsin:TyrR protein, 1:200 by weight) were used to analyze ligand-mediated conformational changes. Higher levels of trypsin (1:11 by weight) were used to observe the protective effects of ATP. Each set of digestion studies was carried out in parallel using a stock solution of trypsin from the same vial. Identical conditions with respect to volume, buffer components, and protein concentration were employed in each set of digestions. The only variable factor was the nature of the TyrR ligands.

Initially, a series of four digestions (Fig. 2) were carried out to test whether ligands affected the susceptibility of the TyrR protein to trypsin. In the absence of any cofactors, the 58-kDa TyrR protein (Fig. 2A, lane 0) was gradually converted into two major trypsin-resistant fragments of 22 and 31 kDa (Fig. 2A). The half-life of the TyrR protein was about 16 min. Complete conversion of the TyrR protein to stable subfragments of 22 and 31 kDa required about 2 h (data not shown). In the presence of tyrosine (500 μM) (Fig. 2B), the pattern of digestion resembled that which was observed in the absence of ligands (Fig. 2A). As before, two main fragments of 22 and 31 kDa were generated. Under the above two conditions, a minor species of 36 kDa was also observed early in the course of trypsin digestion. The nature of this polypeptide will be explained later. In the presence of ATP (200 μM) (Fig. 2C), the TyrR protein was gradually degraded into the same two species as before. However, a new species, (53 kDa) was observed in the early stage of this digestion. The half-life of the TyrR protein in the presence of ATP was 4–8 min, slightly less than the previous two cases. In the presence of both ATP (200 μM) and tyrosine (500 μM) (Fig. 2D), the 22- and 31-kDa subfragments were still the two main products. A 53-kDa polypeptide, identical with that observed in the presence of ATP, was also detected, as was a species of 36 kDa at digestion times of 2 and 4 min (Fig. 2D). In the presence of both ligands, the conversion of the 58-kDa TyrR protein to fragments of 22 and 31 kDa was much more rapid (t1/2, 2–4 min) than in their absence (Fig. 2A).

An identical result was obtained when phenylalanine (2 mM) was substituted for tyrosine. Leucine (2 mM), provided in place of tyrosine, had no effect on the course of trypsin digestion (data not shown). The initial studies led to the following conclusions. 1) Two major core fragments of the TyrR protein, of 22 and 31 kDa, were resistant to cleavage by low levels of trypsin. 2) Tyrosine alone altered neither the kinetics nor the pattern of trypsic digestion of the TyrR protein.

**FIG. 1.** Amino acid sequence of the TyrR protein (Cornish et al., 1986). The features of particular relevance to the present study (see text) are a 22-kDa N-terminal domain (residues 1–198) operationally defined by the trypsin-sensitive peptide bond (A) connecting Arg30 and Glu193, a 31-kDa second domain having strong homology to the α2 transcriptional activators (residues 191–467) operationally defined by the trypsin-sensitive peptide bonds A and C connecting Arg30 and Glu193, and Arg57 and Phe668, the presumptive ATP-binding sites (bracketed residues 234–240 and 291–297), and the operator recognition element (bracketed residues 492–502) (Pittard and Davidson, 1991). The peptide bond connecting Lys306 and Leu346 is a minor point of tryptic cleavage (see text and Fig. 6).
were visualized by staining with Coomassie Blue. The arrows refer to the molecular weight (in thousands) of the tryptic fragments. The times (min) when each sample was removed are shown along the top of each panel. Lanes marked 0 show the TyrR protein samples before trypsin was added. Low levels of trypsin (ratio, 1:200) were used in each of the runs. The precise digestion conditions are described under “Experimental Procedures.” A, no ligands; B, tyrosine (500 μM); C, ATP (200 μM); D, ATP (200 μM) plus tyrosine (500 μM).

FIG. 3. Protection of the 31-kDa tryptic fragment of the TyrR protein by ATP. Samples, collected as described under “Experimental Procedures,” were subjected to 10% SDS-PAGE. The resulting fragments were visualized by staining with Coomassie Blue. Arrows refer to the molecular weights (thousands) of the tryptic fragments. Numbers along the top of each gel indicate the time (min) when samples were taken. Lanes marked 0 were loaded with TyrR protein samples taken before trypsin was added. High levels of trypsin (ratio, 1:1) were employed in this study. Digestion conditions are described under “Experimental Procedures.” A, no ligands; B, with ATP (200 μM).

The data are consistent with a previous report that tyrosine cannot bind to the TyrR protein in the absence of ATP (Argyropoulos, 1989). 3) A tryptic fragment of 53 kDa was liberated from the TyrR protein when digestion was carried out in the presence of ATP. 4) The rate of conversion of the TyrR protein to fragments of 22 and 31 kDa was accelerated when both ATP and tyrosine were present.

ATP Protection—In a previous study (Argyropoulos, 1989), it was shown that ATP binds to the TyrR protein. By computer analysis, two presumptive binding sites (residues 234-240 and 291-297) were found in the TyrR protein (Pittard and Davidson, 1991; Fig. 1). If ATP binds to the central region of the TyrR protein, this event might affect the cleavage of nearby trypsin-sensitive bonds. To investigate this possibility, two parallel digestions were carried out at a concentration of trypsin 20 times higher than before (trypsin:TyrR = 1:11).

In the absence of ATP, the TyrR protein was rapidly converted to two species (22 and 31 kDa) (Fig. 3A). The 22-kDa fragment was relatively resistant to further cleavage by trypsin. However, the 31-kDa fragment was completely degraded after 8 min. The half-life of the 31-kDa species was 2-4 min. A new species, of 23 kDa, arose from the 31-kDa subfragment of the TyrR protein. The 23-kDa fragment was itself highly susceptible to further tryptic hydrolysis. This experiment suggested that the 31-kDa tryptic fragment of the TyrR protein, in the absence of ATP, was susceptible to further attack by trypsin when this protease was present at the appropriate concentration. In the presence of ATP (200 μM), the 31-kDa subfragment was resistant to hydrolysis by high concentrations of trypsin (Fig. 3B). The half-life of the 31-kDa fragment was greater than 1 h. Even after incubation for 2 h, substantial amounts of undigested 31-kDa fragment remained (Fig. 3B). The stability of the 22-kDa fragment in the presence of trypsin was unaffected by ATP. The susceptibility to tryptic hydrolysis of 31-kDa fragment was unaffected by tyrosine (500 μM) (data not shown); when ATP (200 μM) and tyrosine (500 μM) were both present, the course of hydrolysis by trypsin was indistinguishable from that observed in the presence of ATP (data not shown). These data indicate that the 31-kDa tryptic fragment of the TyrR protein, although susceptible to cleavage at high concentrations of trypsin in the absence of ligands, was protected by ATP from trypsin attack. The 22-kDa tryptic fragment was very resistant to digestion by trypsin, even when the ratio of trypsin to the TyrR protein was as high as 1:11.

Effect of Poly(dI-dC)—The behavior of the TyrR protein

FIG. 2. Effects of different ligands on the kinetics of trypsin digestion of the TyrR protein. Samples were collected as described under “Experimental Procedures.” The fragments were separated by 10% SDS-PAGE, and the resulting polypeptides were visualized by staining with Coomassie Blue. The arrows refer to the molecular weight (in thousands) of the tryptic fragments. The times (min) when each sample was removed are shown along the top of each panel. Lanes marked 0 show the TyrR protein samples before trypsin was added. Low levels of trypsin (ratio, 1:200) were used in each of the runs. The precise digestion conditions are described under “Experimental Procedures.” A, no ligands; B, tyrosine (500 μM); C, ATP (200 μM); D, ATP (200 μM) plus tyrosine (500 μM).
during the early steps of purification (see "Experimental Procedures") as well as specific, segments of DNA. To investigate whether the TyrR protein alters its conformation upon hind docking to DNA, two parallel digestions at low levels of trypsin were carried out in the presence or absence of poly(dI-dC). This synthetic DNA-like polymer was chosen because the amounts of DNA that would have been required for complete binding of the TyrR protein precluded the use of material containing authentic TyrR boxes. In the absence of poly(dI-dC), the TyrR protein was gradually converted into two major fragments of 22 and 31 kDa (Fig. 4A). A minor species of 36 kDa was observed transiently in the early stage of the digestion (Fig. 4A). In the presence of poly(dI-dC), the TyrR protein was rapidly converted into a species of 53 kDa (Fig. 4B), previously observed (Fig. 2, C and D) in the presence of ATP. Trypsin digestion in the presence of poly(dI-dC) led to the accumulation of a previously undetected minor species of 45 kDa (Fig. 4B). The final trypsin-resistant core fragments in this digestion were also polypeptides of 22 and 31 kDa (Fig. 4B). These data suggested that the binding of poly(dI-dC) led to a conformational change in the TyrR protein. In particular, the yield of the 53 kDa tryptic fragment was greatly increased, as compared with the pattern of digestion without poly(dI-dC) (Fig. 4A). Moreover, an intermediate species of 45 kDa was observed only in the presence of poly(dI-dC).

**Chemical Identity of the Tryptic Fragments of the TyrR Protein**

To characterize the tryptic digestion products of the TyrR protein, each fragment was electrophoretically purified and subjected to sequential Edman degradation analysis. The identity and amounts of the first 6 amino acids at the N terminus of each fragment are presented in Table I. The N-terminal amino acid sequence of the 22-kDa core tryptic fragment (Table I) proved that this species originated from the N-terminal end of the TyrR protein. There are three possible sites of trypsin cleavage (R190, R197, and R206) that could be the C terminus of this fragment. The apparent molecular weight of 22 kDa closely matches the estimated molecular weight of a polypeptide containing amino acid residues 1-187 or 1-190 of the TyrR protein. This observation makes it likely that the 22-kDa fragment terminates at R197 or R206. The N-terminal amino acid sequence of the other main core tryptic fragment (31 kDa) was QLQNVA (Table I), which is that of residues 191-196 of the TyrR protein. This result suggests that R190-Q191 is the preferred site of trypsin cleavage in the formation of the 22- and 31-kDa fragments of the TyrR protein.

The apparent molecular weight of the 31-kDa fragment was appreciably lower than the calculated molecular weight (35,974) of a polypeptide containing amino acid residues 191-513, making it unlikely that the 31-kDa fragment included the C terminus of the TyrR protein. The minor trypsin digestion product of 36 kDa (Fig. 2) had the same N-terminal sequence as the 31-kDa fragment (Table I). This polypeptide, in all likelihood, contains amino acid residues 191-513. Therefore, the 31-kDa core fragment must be derived from segment 191-513. To identify the C-terminal end of the 31-kDa fragment, its precise molecular weight was determined by electrospray mass spectrometry. The 31-kDa preparation contained two distinguishable fragments (Fig. 5). The molecular weight of the major form was 30,617, which is very close to the predicted molecular weight (30,597) of segment 191-467 of the TyrR protein. The molecular weight of the minor form was 31,044, which matches the estimated molecular weight (31,029) of amino acid residues 191-470. These data suggest that the TyrR protein consists of two principal domains, namely an N-terminal domain (approximately residues 1-190), and a second domain (residues 191-467).

To detect small polypeptides that might reveal the existence of a domain structure within the C-terminal region, a careful series of experiments deliberately aimed at detecting such a species were conducted. Modifications to the standard protocol included the use of trichloroacetic acid as a protein precipitant and varying the nature of the SDS-PAGE system (data not shown). In no case were trypsin digestion products of 5 kDa or smaller, which might indicate an ability of the C terminus of the TyrR protein to exist as a stable trypsin-resistant species, observed.

The 53-kDa fragment that was detected in tryptic digests conducted in the presence of ATP or poly(dI-dC) had the same N terminus as the full-length TyrR protein (Table I). The near identity in molecular weight between the 53-kDa fragment and the combined molecular weight of the 22 and 31-kDa species supports the notion that the 53-kDa fragment includes residues 1-467. Owing to the difficulty of purifying it, the precise molecular weight of the 53-kDa fragment could not be determined by electrospray mass spectrometry.

**DISCUSSION**

**Ligand Effects**—The effects of ligands on the pattern of tryptic digestion of the TyrR protein are summarized in Fig. 6. In the absence of any ligands, the major cleavage sites are A and C; B is a minor cleavage site. At low levels of trypsin, the 36-kDa tryptic peptide was not observed in the presence of ATP (Fig. 2C). However, a 53-kDa fragment was readily detectable. Evidently, the binding of ATP enhances the cleavage rate of the R

![Fig. 4. Effect of poly(dI-dC) on the tryptic digestion pattern of the TyrR protein.](image-url) Samples were analyzed as described in the legends to Figs. 2 and 3. Arrows refer to the molecular weights (thousands) of the tryptic fragments. Numbers along the top of each panel indicate the time (min) when samples were taken. Lanes marked 0 were loaded with TyrR protein samples untreated with trypsin. Low levels of trypsin (1:200) were employed in each case. Digestion conditions are described under "Experimental Procedures." A, no ligands; B, with poly(dI-dC) (20 μg/200 μl).
TABLE I

N-terminal sequence analysis of tryptic peptide fragments derived from the TyrR protein

For a description of the isolation of the peptides whose analysis is described, see “Experimental Procedures.”

| Edman deg.- | 53-kDa | 46-kDa | 36-kDa | 31-kDa | 23-kDa | 22-kDa |
| radiation | PTH-derivative | Amount | PTH-derivative | Amount | PTH-derivative | Amount | PTH-derivative | Amount | PTH-derivative | Amount | PTH-derivative | Amount |
| cycle amino acid | pmol | pmol | pmol | pmol | pmol | pmol | pmol | pmol | pmol | pmol | pmol | pmol |
| 1 | M | 18 | M | 2 | Q | 22 | Q | 32 | Q | 68 | M | 370 |
| 2 | R | 17 | R | 2 | L | 22 | L | 26 | L | 23 | R | 323 |
| 3 | L | 13 | L | 2 | Q | 20 | Q | 33 | Q | 19 | L | 373 |
| 4 | E | 15 | E | 2 | N | 22 | N | 24 | N | 34 | E | 239 |
| 5 | V | 22 | V | 1 | V | 16 | V | 23 | V | 14 | V | 329 |
| 6 | F | 29 | F | 2 | A | 16 | A | 14 | A | 16 | F | 305 |

When both ATP and tyrosine were present, the conversion of the TyrR protein to fragments of 22 and 31 kDa was enhanced. Under this condition, fragments of 53 and 36 kDa also became detectable (Fig. 2D). These data suggest that the binding of ATP and tyrosine accelerates the rate of cleavage of both the R<sup>190</sup>-Q<sup>191</sup> and R<sup>467</sup>-F<sup>468</sup> peptide bonds. In all likelihood, the binding of ATP to the TyrR protein leads to greater solvent exposure of the R<sup>467</sup>-F<sup>468</sup> peptide bond; after this has happened, tyrosine binding leads to a conformational change that makes the R<sup>190</sup>-Q<sup>191</sup> peptide bond more accessible.

Another effect of ATP was the protection of the 31-kDa second domain of the TyrR protein from attack by high levels of trypsin (Fig. 3B). This result is consistent with the predicted locations of two presumptive ATP-binding sites that exist within this region of the TyrR protein. When the TyrR protein was exposed to high levels of trypsin in the absence of ATP, a 23-kDa fragment was generated from the 31-kDa fragment (Fig. 3A). This 23-kDa species had the same N terminus as the 31-kDa fragment (QLQNVA, Table I). From its estimated molecular weight, the 23-kDa fragment probably contains amino acid residues 191–398 (Fig. 6). This segment could well be a subdomain of the second domain.

When trypsin digestion of the TyrR protein was carried out in the presence of poly(d1-dC), there were two major consequences. First, there was substantial accumulation of a 53-kDa fragment and a comparable reduction in the levels of the 22- and 31-kDa fragments (Fig. 4, A and B). The second consequence of digestion in the presence of poly(d1-dC) was the appearance of a new polypeptide of 45 kDa and an absence of the 36-kDa fragment (Fig. 4, A and B). The 45-kDa fragment had the same N terminus as the full-length TyrR protein (Table I). These observations suggest that poly(d1-dC) greatly accelerates the digestion rate of the R<sup>467</sup>-F<sup>468</sup> peptide bond and greatly diminishes the digestion rate of the R<sup>190</sup>-Q<sup>191</sup> peptide bond. The production of the 53-kDa fragment, attributable to hydrolysis at site C, occurs prior to digestion at site A (Fig. 6). The 45-kDa fragment arises from a hydrolytic event at minor site B prior to cleavage at site A (Fig. 6). Evidently, upon binding to poly(d1-dC), conformational change occurs within the TyrR protein such that site C becomes the most solvent exposed site, and site A becomes much less exposed, in comparison to unliganded TyrR protein.

To a first approximation, the poly(d1-dC) effect (Fig. 4) can be considered to mimic the effect of the natural TyrR boxes. In a previous spectral study, the immunity repressor of phage λ was shown to undergo identical conformational changes either upon interaction with specific operator DNA or with DNA-like polymers (Saha et al., 1992). A similar finding was made in a study of the cyclic AMP-binding protein (Angulo and Krakow, 1986). In another study, poly(d1-dC) was shown to stimulate the ATPase activity of NtrC, a α<sup>4+</sup>-specific transcriptional activator, in a manner similar to that of authentic target DNA for this protein (Austin and Dixon, 1992). It is therefore not unreasonable to suppose that the binding of the TyrR protein to poly(d1-dC) is associated with a conformational change that is similar to that which occurs normally during transcriptional regulation.

Both ATP and poly(d1-dC) led to a greater solvent exposure of the R<sup>467</sup>-F<sup>468</sup> peptide bond. The hydrolysis of the R<sup>190</sup>-Q<sup>191</sup> peptide bond increased in the presence of ATP plus tyrosine and was diminished in the presence of poly(d1-dC). These data raise the possibility that the accessibility of the R<sup>190</sup>-Q<sup>191</sup> peptide bond to trypsin reflects conformational events that occur when this protein exercises its transcriptional regulatory function.

Domain Structures—Our results suggest that the TyrR protein is organized as two distinct domains, encompassing about 91% of the amino acid residues. The two substructures consist of an N-terminal domain (approximately residues 1–190) and a second domain (residues 191–467). The remaining 9%, at the C terminus, containing the operator recognition elements, was never observed as a discrete tryptic peptide, despite numerous attempts to detect a species of the predicted size.

FIG. 5. Mass spectrometric analysis of the 31-kDa tryptic fragment of the TyrR protein. For details on the preparation and analysis of this species, see text.
FIG. 6. Diagrammatic representation of effects of ligand on the pattern of trypsin digestion of the TyrR protein. Solid arrows (A and C) refer to the two major tryptic cleavage sites. The dashed arrow refers to a minor tryptic cleavage site within the second domain. The N-terminal domain (residues 1–190) is darkly shaded. The second domain (residues 191–467) is lightly shaded. The darkened area on the right represents the DNA-binding element (residues 482–502). The two vertical bars in the second domain identify presumptive ATP-binding sites (see Fig. 1). The TyrR protein (58 kDa) and tryptic fragments derived from it are drawn as horizontal lines. Numbers on the right show the molecular weights of the tryptic fragments that were observed. +, accelerated rate of digestion in the presence of ligands; −, reduced rate of digestion; P, protection against trypsin digestion; O, no effect.

The second domain of the TyrR protein shows substantial identity with the so-called "central" domains of the activators specific for the $\sigma^4$ form of RNA polymerase (Fig. 1) despite the fact that the regulatory promoters utilize the $\sigma^70$ form. These segments of the $\sigma^4$ activators bind ATP. In one such activator, NtrC, ATP hydrolysis accompanies the conversion of the RNA polymerase-promoter complex from the "closed" to the "open" form, thereby enabling transcription to proceed (Popham et al., 1989). Two ATP-binding sites were predicted to lie within the second domain of the TyrR protein. Our observation that ATP could prevent a trypsin cleavage event within the second domain of the TyrR protein confirms and extends previous in vitro studies wherein it was demonstrated that ATP binds to the TyrR protein (Argyropoulos, 1989). Despite the accumulated evidence that the second domain of the TyrR protein binds ATP, the in vivo physiological role of this event is unknown.

Although the first 190 amino acids of the TyrR protein are organized in the form of a structurally discrete domain, the function of this segment, or how it interacts with the remainder of the protein, has not been established. The role of the N-terminal domains of the $\sigma^4$ activators is to participate in regulatory interactions with other factor(s). For example, the N-terminal domain of NtrC contains a residue (Asp$^64$) that is phosphorylated in a reaction catalyzed by NtrB. NtrC must be phosphorylated in order to carry out its activation function (Cui and Somerville, 1993a, 1993b). By analogy with the NtrC case, it is reasonable to suggest that the role of the N-terminal domain of the TyrR protein is to originate transcriptional regulatory signals that are transmitted via the second domain to an operator recognition region.

The $\sigma^4$-specific activators are thought to contain glutamine-rich connectors between their N-terminal and central domains (Wootton and Drummond, 1989). These connectors are considered to play a role in transmitting regulatory signals between domains. The insertion of numerous extra amino acids into these connectors does not lead to a loss of function (Wootton and Drummond, 1989). From an inspection of the amino acid sequence of the TyrR protein, it is likely that a similar connector (residues 186–200) is situated between the two major domains of the TyrR protein. Such linkers are also present in certain $\sigma^70$-specific activator proteins that are members of two-component bacterial regulatory systems (Wootton and Drummond, 1989).

The concept that large proteins may contain multiple, distinct, independently folding structural regions was enunciated about 20 years ago (Wetlaufer, 1973). One serviceable definition for a protein domain is "a structurally independent compact globular region consisting of a continuous stretch of polypeptide chain, from 100–200 amino acids long" (Coggins and Hardie, 1986). Structural domains of proteins are frequently resistant to digestion by low levels of certain proteases. Limited proteolysis has not previously been employed to study either the TyrR protein (a $\sigma^70$-specific transcriptional regulatory factor) or any of the homologous prokaryotic activator proteins, all of which happen to be specific for the $\sigma^4$ form of RNA polymerase. It has been suggested that this family of proteins is organized in the form of three structural domains, namely an N-terminal domain, a central domain, and a C-terminal domain (Drummond et al., 1986; Nixon et al., 1986). We were able to detect only two domain structures in the TyrR protein. The C-terminal region, rich in trypsin-sensitive peptide bonds, appears to be loosely folded. Our evidence does not exclude the possibility that amino acid residues 468–513 are organized as a distinct substructure. However, by the criterion of limited proteolysis, the C terminus of the TyrR protein fails to qualify as a domain. The same situation may well apply to NtrC, DctD, NiF, and other members of this family of proteins. The present study is the first chemical demonstration of an authentic domain structure in any member of the family of prokaryotic transcriptional activators homologous to TyrR. Our results provide a chemical basis for localizing domain boundaries. This information may be useful in the future functional analysis of proteins that have structural homologies to the TyrR protein.

Acknowledgments—For advice and technical assistance, we thank Weiping Yang and Tiee-Leou Shieh. For assistance with computational tasks, we thank Ihor Skrypka. For careful mass spectrometric analyses, we thank Kristine Swiderek of the City of Hope National Cancer Institute. We are grateful to Virginia Heatwole, Guo-Ping Zhao, and Gaociao Zhou for advice and comments during the course of this work and the preparation of the manuscript and to Janell Rex for helpful discussions.

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