CHARACTERIZATION OF THE LAC REPRESSOR SPECIES PRODUCED BY LIMITED TRYPTIC CLEAVAGE

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SUMMARY: Tryptic cleavage of native lac repressor under very mild conditions has been found to yield preparations suitable for detailed physical and chemical analysis. Sephadex G-200 chromatography of the digest produces one main protein peak followed by small peptides. The protein from the main peak was analyzed by automated Edman degradation and revealed two unique cleavage sites, one at residue 51 and the other at 59. The tryptic core protein under native conditions is tetrameric and exhibits a circular dichroism spectrum similar to that of native lac repressor.

INTRODUCTION

The lac repressor regulates lactose metabolism in E. coli at the level of transcription by binding to the operator region of the lac operon (1). Studies of repressor in this laboratory (2,3) have been directed toward understanding the structural details of its different conformational states. In the present investigation, we studied the selective removal of the amino-terminal region. Starting with the procedure of Platt et al. (4), we have determined conditions for limited tryptic hydrolysis which yield repressor species of sufficient homogeneity for physicochemical studies. Furthermore, the pronounced proteolytic susceptibility of certain areas near the amino-terminus suggest some important structural details of the lac repressor.

MATERIALS AND METHODS

Lac repressor was prepared from an overproducing strain of E. coli, M 96 (origin, Jeffrey Miller), according to the procedure of Platt et al. (4).

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Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (5) was used to assess the purity of repressor and digestion products. Sedimentation velocity and equilibrium data were determined with a Model E analytical ultracentrifuge equipped with scanner and interference optical systems. According to the procedure of Babul and Stellwagen (6), the extinction coefficient for the repressor was $E^{1\%}_{280\ nm} = 5.9 \pm 0.1$, and for the tryptic core, $E^{1\%}_{280\ nm} = 5.7 \pm 0.5$. Circular dichroism spectra were recorded on a Jasco J-40 Spectropolarimeter with cells as small as 0.2 mm in path length, and absorption spectra were recorded on Cary 14 and 16 spectrophotometers.

Proteolysis was performed on repressor dialyzed against 0.1 M NH_4HCO_3 at 1°C and centrifuged before the concentration was measured (4 to 8 mg/ml). TPCK*-trypsin (Worthington Biochemical Corp.), dissolved in 10^{-3} M HCl at 0°C, was added to 0.05% (w/w), and the mixture incubated at 5°C for 2 hours. The reaction was terminated by adding 10µl of phenylmethanesulfonylfluoride (Sigma Chemical Co., 30 mg per ml ethanol) per ml of digest, and the mixture was then immediately applied to a 1.5 x 85 cm column of Sephadex G-200 equilibrated with 0.1 M NH_4HCO_3 at 1°C. Yields of about 75 to 80% were obtained, relative to the maximum core protein product expected.

The protein represented by the main peak was pooled, reduced, carboxymethylated (7), and used for sequence studies. It was subjected to automated Edman degradation (8) on a Beckman Model 890 C Sequencer; the fast quadrol double cleavage program provided by the manufacturer was used (9). The PTH+-amino acids were identified on a Beckman Model 65 gas chromatograph (10), and confirmed by thin layer chromatography (11) and hydrolysis with HI (12). Carboxypeptidase A and B (Worthington Biochemical Corp.) treated with DFP† were used to determine whether cleavage had occurred interior to the carboxyl-terminus of the molecule (13). All amino acid analyses were done on a Beckman 121 Amino Acid Analyzer (14).

*TPCK = L-(1-Tosylamido-2-phenyl) ethyl chloromethyl ketone
+PTH = phenylthiohydantoin
†DFP = diisopropyl phosphorofluoridate

RESULTS AND DISCUSSION

The elution profile of lac repressor cleaved with 0.05% trypsin and chromatographed on Sephadex G-200 is shown in Fig. 1a. The 280 nm absorb-

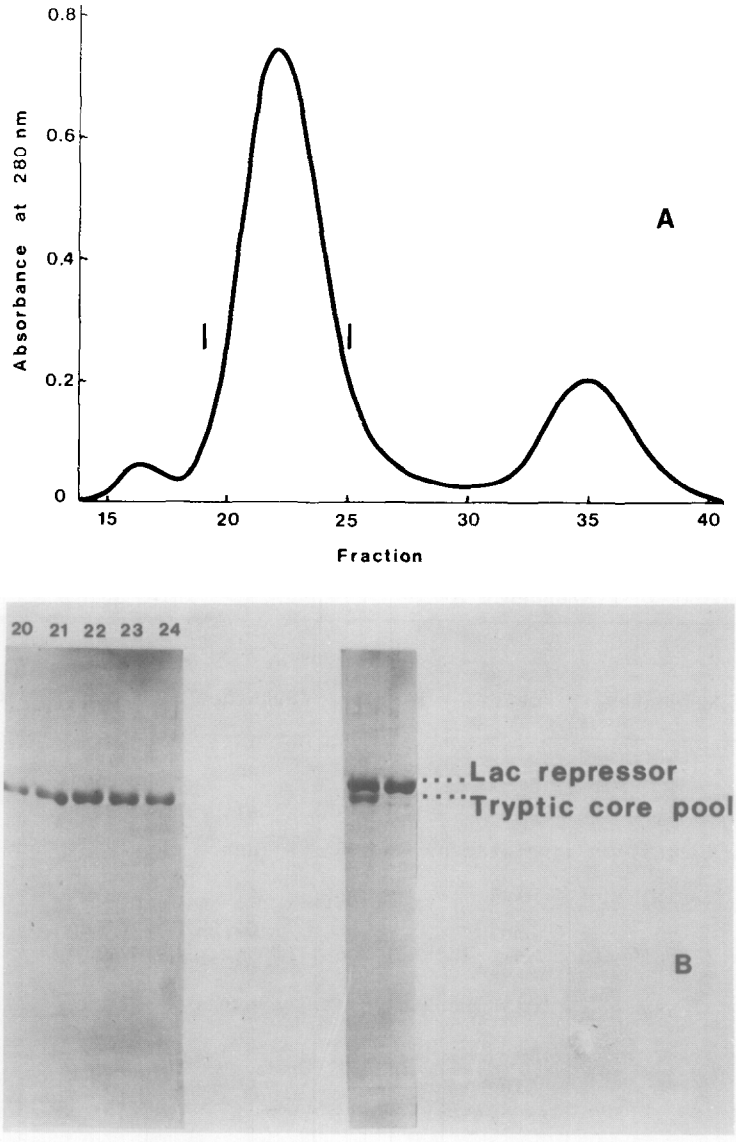

Figure 1. (a) Gel chromatography of 6.5 ml repressor digest (4.6 mg/ml) on Sephadex G-200 in 0.1 M NH_4HCO_3 (1.5 x 85 cm column), with fractions of 3.6 ml collected; vertical lines indicate fraction pooled for subsequent sequence analysis. (b) SDS gel electrophoretogram of eluted fractions of tryptic core from chromatography of Fig. 1a, pooled core mixed with repressor, and lac repressor alone; 12.5% polyacrylamide slab gels were used (5).

ance reveals a large, symmetric peak which was pooled for chemical studies of the tryptic core protein; a minor leading peak contains aggregate and the final broad peak comprises tyrosine-containing amino-terminal peptides. SDS gel electrophoresis of individual tryptic core fractions reveals a single band with molecular weight in the range of 30,000 to 35,000 (Fig. 1b). If gels were heavily loaded, trace bands could be observed below 30,000, which contrasts with the results of Platt et al. (4). They cleaved repressor under more drastic conditions of 1% trypsin at 37°C for 20 to 30 min and found three main bands between 28,000 and 33,000 molecular weight.

The chromatographically pure tryptic core was submitted to sequence analysis for determination of its cleavage sites, and the results are given in Table 1. Two amino acids were released by each cycle of Edman degrada-

TABLE 1

AUTOMATED EDMAN DEGRADATION OF LAC REPRESSOR CORE PROTEIN

	Sequence A			Sequence B		
Residue #	Sequence #	Assign-ment	n Moles	Sequence #	Assign-ment	n Moles
1	52	Val	96.6	60	Gln*	--
2	53	Ala	68.8	61	Ser*	111.4
3	54	Gln*	--	62	Leu	107.8
4	55	Gln*	--	63	Leu	194.7
5	56	Leu°	--	64	Ileu°	177.1
6	57	Ala	71.7	65	Gly	51.8
7	58	Gly	43.6	66	Val	62.2
8	59	Lys†	--	67	Ala	63.9
9	60	Gln*	--	68	Thr*	38.6
10	61	Ser*	--	69	Ser*°	67.0
11	62	Leu	60.8	70	Ser*	85.1
12	63	Leu°	--	71	Leu°	95.8

* These values are estimates, identified as the trimethylsilyl derivatives and confirmed by thin layer chromatography and hydrolysis with HI.
† Confirmed by thin layer chromatography.
° Recorded as a total.

tion, an indication of two main amino-termini in the protein sample. A comparison of residues released with the known sequence of lac repressor (15) indicated that a unique alignment of residues is obtained that corresponds to core polypeptides beginning at valine 52 and glutamine 60. In a few instances minor sequences were detected which could probably be accounted for by the presence of trace amounts of species of lower molecular weight. Thus trypsin hydrolyzes native repressor primarily at arginine 51 and lysine 59 to produce species of molecular weight 31,500 and 30,700. Platt and his coworkers (4) had previously shown cleavage at lysine 59 by analysis of released peptides, but end groups were not measurable. From the data for carboxypeptidase A and B digestion given in Table 2, it is evident that only moderate

TABLE 2

DIGESTION OF TRYPTIC CORE OF LAC REPRESSOR BY CARBOXYPEPTIDASE A AND B

Amino Acid (n mole/ml	Time (hours) 0.5	1.0	2.0	4.0
Glutamine*	9.8	20.2	27.7	36.0
Alanine	4.8	7.2	9.7	16.8
Leucine	trace	6.0	10.7	22.5
Methionine	-	-	-	7.4
Valine	-	-	-	6.0
Arginine†	trace	N.D.	10.5	N.D.

*Glutamine elutes in the position of serine. Hydrolysis in 6N HCl at 110°C yields glutamic acid, confirming the presence of the amide.

†Arginine was determined only at 0.5 and 2.0 hour. Incubation was in 0.1 M NH_4HCO_3 pH 7.9 at 37°C. The reaction was terminated by adding 1 drop of 6N HCl followed by lyophilization. After resuspension in 0.01 N HCl the mixture was filtered to remove precipitated protein and the supernatant applied to the columns of the amino acid analyzer.

tryptic cleavage occurs at the carboxyl-terminal region of repressor under our conditions, whereas it is more extensive with more severe conditions (4,15). These results of carboxyl-terminal analyses indicate that intact repressor core protein is present, and tryptic cleavage is principally at arginine 338 and 342.

Our native tryptic core was found to be tetrameric based on a sedimentation coefficient of 6.0 S (determined at 20°C in 0.1 M NH_4HCO_3, at a protein concentration of 0.7 mg/ml), and on its elution from Sephadex G-200 as a single peak slightly after that of native repressor. In contrast, conditions approximating those described by Platt et al. (4) gave two closely overlapping main peaks upon Sephadex G-200 chromatography of the digest, which suggests the presence of other than native tetramers. Since our species exhibited a tendency to aggregate in 0.1 M NaCl + 10^{-4} M dithiothreitol, pH 7.6, as evidenced by curvature of its sedimentation equilibrium plot, an exact mass for native tryptic core was unobtainable; however, the minimum weight average molecular

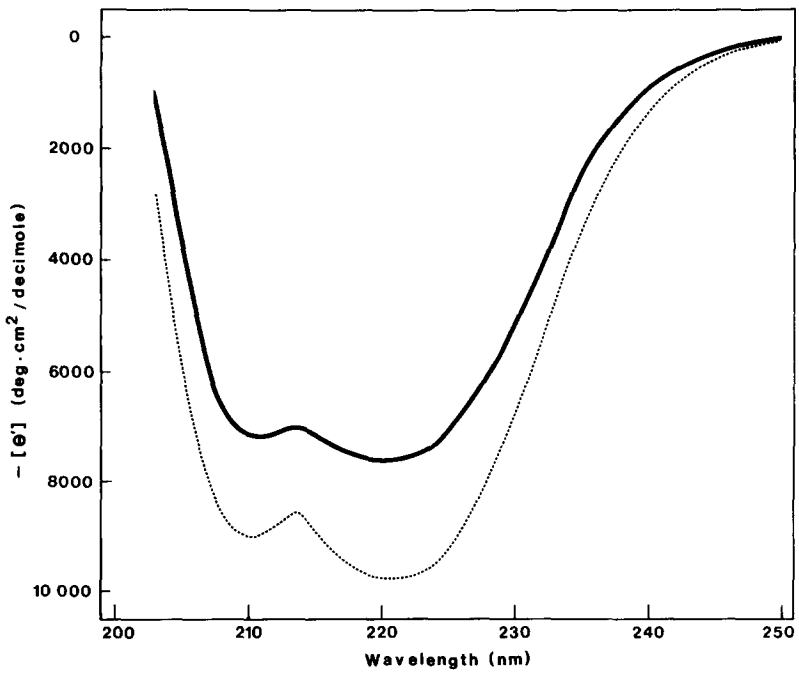

Figure 2. Circular dichroism spectra of lac repressor (dotted line) and its tryptic core (solid line), in 0.1 M NaCl, pH 7.8.

weight of about 145,000 observed is compatible with a tetrameric structure.

Retention of native repressor structure by the tryptic core species was shown by its complete immunological cross reactivity with repressor when monitored by immunodiffusion against either anti-repressor or anti-tryptic core rabbit serum. Circular dichroism spectra of the repressor and the tryptic core, shown in Fig. 2, reveal double minima at 210 nm and 220 nm, with the main difference being more shallow minima for the tryptic core spectrum. This suggests loss of α-helix (16), and is likewise consistent with the maintenance of most native repressor structure. The tryptic core was also found to bind ^{14}C-isopropylthiogalactoside with a micromolar dissociation constant, as was previously reported (4).

This investigation reveals that lac repressor can be specifically cleaved with trypsin at arginine 51 and lysine 59 to yield tetrameric species lacking the peptides amino-terminal to residues 52 and 60. This unusual susceptibility to cleavage suggests a partially exposed amino-terminal structure, in agreement with genetic evidence that its sequence up to alanine 53 is crucial to operator binding (17,18).

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