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INTERACTION OF SYNTHETIC NH_2-TERMINAL FRAGMENTS OF BACTERIOPHAGE λ CRO PROTEIN WITH NUCLEIC ACIDS

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SUMMARY: It was found that the synthetic NH_2-terminal 22-residue fragment of bacteriophage λ cro protein and a covalently dimeric analog of this fragment bound to both double-stranded and single-stranded DNA. Affinity chromatography and UV difference spectroscopy showed that interaction with λ DNA was stronger than that with calf thymus DNA. The dimeric peptide had higher affinity for nucleic acids than the monomeric peptide indicating that cro protein functions as an oligomer. Both the monomeric and the dimeric peptide exhibited specificity for binding poly(G) and the Eco RI fragment of λ DNA that contains the immunity region. The nucleic acid-binding properties of the 2 synthetic NH_2-terminal cro protein fragments could be explained by the proposed folding of the NH_2-terminus of cro protein.

Genetic evidence had indicated that activity as well as specificity of cro protein, a 66-residue negative regulator of bacteriophage λ, may reside in the NH_2-terminal 22-residue portion (G. Hobom, personal communication). As specific binding to operator DNA was also found for the NH_2-terminal 59-residue fragment of lac repressor (1,2) and for the NH_2-terminal 92-residue domain of λ repressor (3) and as the sequences of the cro gene (4) and the cro gene product (5) were known, we synthesized the NH_2-terminal 22-residue fragment of cro protein to study its nucleic acid-binding properties. Since the 22-residue fragment aggregated in solvents containing ethyl acetate or n-butanol, possibly reflecting an intrinsic tendency of intact cro protein to form oligomers similar to lac repressor (6) and λ repressor (7), we synthesized in addit-

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Abbreviation used: TCA, trichloroacetic acid.

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ion a covalently dimeric analog of the 22-residue fragment.

The NH_2-terminal 22-residue fragment of cro protein had the following amino acid sequence (4,5): Met-Glu-Gln-Arg-Ile-Thr-Leu-Lys-Asp-Tyr-Ala-Met-Arg-Phe-Gly-Gln-Thr-Lys-Thr-Ala-Lys-Asp. In the dimeric analog, the peptide was extended at the COOH-terminus by a cysteine residue which allowed crosslinking of 2 chains by a disulfide bond to give a 46-residue cro protein fragment. Furthermore, Met 12 was replaced by His and Gln 16 was replaced by Glu leaving the overall charge unaltered.

MATERIALS AND METHODS

DNA of bacteriophage λ cI 857 Sam7, calf thymus DNA, tRNA from baker's yeast, and restriction endonuclease Eco RI were obtained from Boehringer Mannheim. Poly(A), poly(G), poly(C), poly(U), and single-strand specific S_1-nuclease were from Sigma. Amino acid derivatives for the peptide syntheses were from Bachem. Peptides were synthesized by the solid phase method (8,9) as described previously (10). Cleavage from the resin and isolation of the products followed the procedure published recently (11). After chromatography on Sephadex G-50, the 22- and the 46-residue peptide were obtained in 60% yield based on the amount of COOH-terminal amino acid originally esterified to the resin. The peptides were purified further by gradient elution from CM-Cellulose (0.01M to 0.5M CH_3COONa, pH 5.5). The yield of the homogeneous products was 30% based on the amounts applied to CM-Cellulose. The 46-residue peptide moved as a sharp spot on TLC (ethyl acetate/acetic acid/pyridine/1M urea, 3:1:5:3, R_F= 0.84) and on thin layer electrophoresis (0.1M pyridine-acetate, 2M urea, pH 5.8; 600V, 50min; mobility, 3.9cm) and had the following amino acid composition (values expected are in parentheses): Asp 4.0(4), Thr 6.0(6), Glu 5.4(6), Gly 2.2 (2), Ala 4.0 (4), Cys 1.8 (2), Met 2.0(2), Ile 1.8(2), Leu 2.0 (2), Tyr 1.8 (2), Phe 2.0 (2), Lys 6.0(6), His 1.8(2), Arg 3.8 (4). The 22-residue peptide was of comparable purity.

RESULTS AND DISCUSSION

UV difference spectroscopy. UV difference spectroscopy showed that 22- and 46-residue fragment interacted with double-stranded and single-stranded DNA. As indicated by Fig.1, the covalently dimeric fragment was 3 times more efficient in changing the spectrum of double-stranded λ DNA and 30 times more efficient in changing the spectrum of single-stranded λ DNA as compared with the monomeric fragment on a molar basis. The absorption minima at 258nm (Fig.1,B and D) suggested that in the presence of the 2 peptides the DNA bases were less exposed. As controls, a synthet-

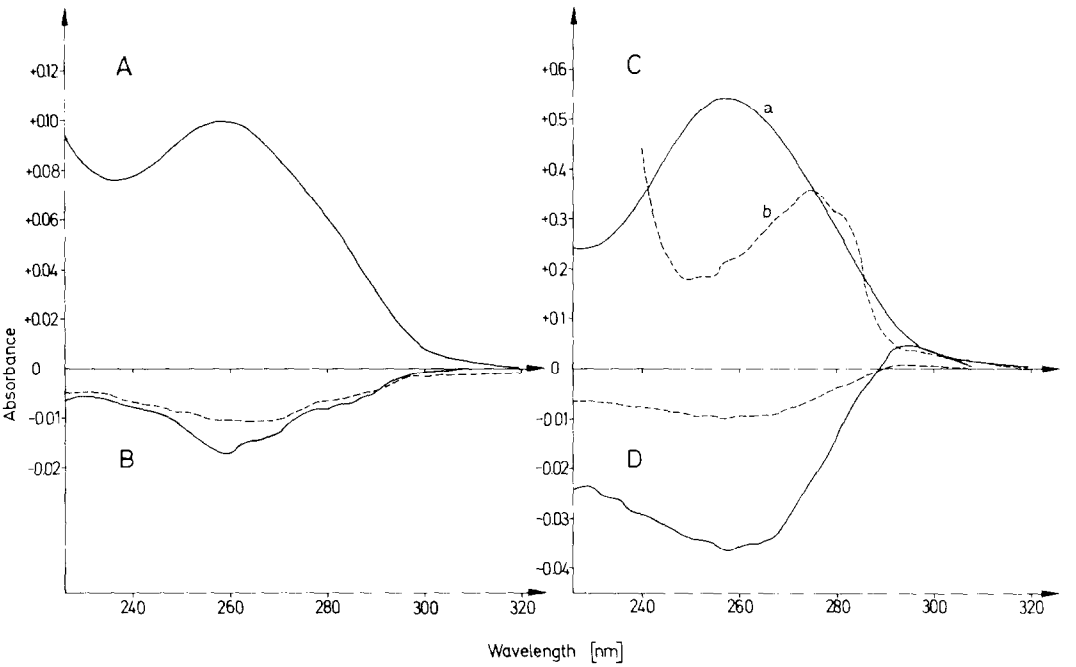

Figure 1: UV difference spectroscopy of double-stranded (A,B) and single-stranded bacteriophage λ DNA (C,D) in the presence of 22- and 46-residue cro protein fragment. Measurements were performed in a Cary 118 spectrophotometer. A; 0.01mg of λ DNA in 1.5ml of buffer, pH 6.0, containing 0.06M NaCl, 0.04M sodium cacodylate, and 0.001M EDTA (d=0.877cm). B; difference spectrum of λ DNA in the presence of a 1.01μM solution (0.004mg/1.5ml) of 22-residue peptide (dashed line) and a 0.49μM solution (0.004mg/1.5ml) of 46-residue peptide (solid line). The peptides were dissolved in the cacodylate buffer, pH 6.0. After mixing peptide and DNA solutions in a tandem cuvette, spectra were recorded against the separate components in a second tandem cuvette (d=0.877cm). C,a; 0.025mg of single-stranded λ DNA in 1.5ml of the cacodylate buffer, pH 6.0. Single-stranded λ DNA was prepared from double-stranded λ DNA by alkali denaturation. b; 22-residue fragment (38μM) in cacodylate buffer, pH 6.0. The spectrum was expanded 5-fold for clarity. The spectrum of the 46-residue fragment was similar. D; difference spectrum of single-stranded λ DNA in the presence of a 2.63μM solution (10.4μg/1.5ml) of 22-residue peptide (dashed line) and a 0.32μM solution (2.6μg/1.5ml) of 46-residue peptide (solid line). The peptides were dissolved in the cacodylate buffer, pH 6.0. The cuvettes used were tandem cuvettes (d=0.877cm).

ic positively charged 26-residue peptide containing Asp, Gly, Ala, Cys, Val, and His residues, and natural insulin were added to double-stranded DNA. The spectrum of the 26-residue peptide-DNA mixture was indistinguishable from that of the separate components whereas that of the insulin-DNA mixture had an absorption maximum at 258nm indicating enhanced exposure of the DNA bases

through partial unwinding of the 2 strands. These studies revealed also that interaction of the 2 peptides with double-stranded λ DNA was more specific than that with double-stranded calf thymus DNA. The maximum absorption difference of λ DNA produced in the presence of the 46-residue fragment was 14 times larger than that of calf thymus DNA and the maximum absorption difference of λ DNA produced in the presence of the 22-residue fragment was 60 times larger than that of calf thymus DNA.

UV difference spectroscopy of poly(A), poly(G), poly(C), and poly(U) in the presence of 46-residue fragment showed that the

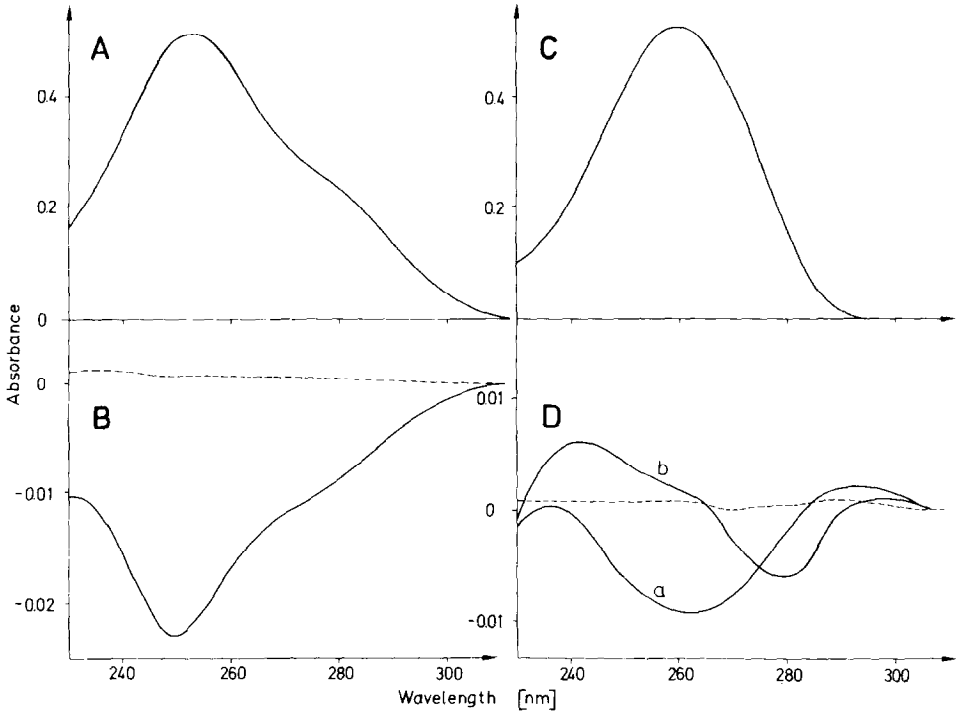

Figure 2: UV difference spectroscopy of poly(G) (A,B) and poly(U) (C,D) in the presence of 46-residue cro protein fragment. A; 0.032mg of poly(G) in 1.5ml of 0.1M cacodylate buffer, pH 6.0. B; difference spectrum (solid line) of poly(G) in the presence of a 2.43μM solution (0.02mg/1.5ml) of 46-residue peptide in the cacodylate buffer. C; 0.033mg of poly(U) in 1.5ml of 0.1M cacodylate buffer, pH 6.0. D; difference spectrum of poly(U) in the presence of a 24μM solution (0.199mg/1.5ml) of 46-residue peptide in the cacodylate buffer immediately after mixing of the 2 components (a) and 30h later (b). At that time the absorption minimum had shifted from 262 to 280nm, and toward shorter wavelengths the absorption had increased. This indicated weak nuclease activity of the 46-residue peptide which was confirmed by the Anfinsen assay on RNA - 46-residue peptide mixtures. A ribonuclease activity of 0.015% was found. The 22-residue fragment was devoid of nuclease activity. Dashed lines are base lines. The cuvettes were the same as in Figure 1.

largest spectral change was obtained with poly(G)(Fig.2,A and B). This was indicative of a specific interaction between 46-residue fragment and poly(G) or G clusters in other nucleic acids. Based on the prediction rules of Chou and Fasman (12) and Levitt (13), the secondary structure of the synthetic cro protein fragments was assumed to be an α-helix interrupted by a turn formed by residues 15-18 (Fig.3). Model building showed that 5 out of the 6 positive charges of the 22-residue peptide and 6 out of the 7 positive charges of one half of the 46-residue peptide were on one side of the helical cylinder allowing electrostatic interactions with the phosphate groups of nucleic acids. Furthermore, the

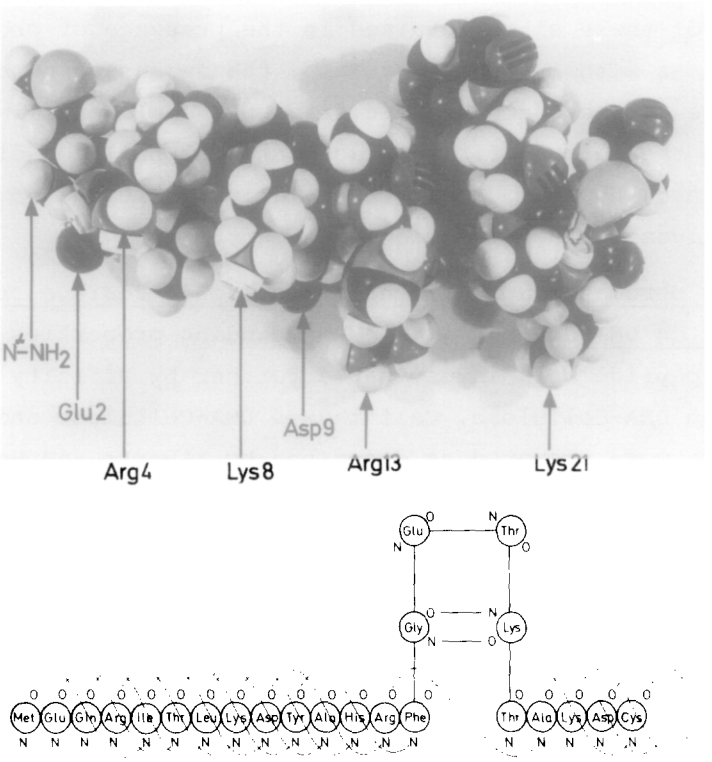

Figure 3: Proposed secondary structure of one half of the 46-residue fragment and of the 22-residue fragment (bottom). The α-helix is interrupted by a turn formed by residues 15-18. Thin lines connecting letters N and O indicate hydrogen bonds between NH and CO groups of the corresponding amino acid residues. In the space-filling model (top), the N^{α}-NH_2 group and those basic residues that have their side chains on the same side of the helical cylinder are marked (His 12 of the 46-residue fragment is above Arg 13). Also shown are the side chains of Glu 2 and Asp 9 which are possibly involved in the specific recognition and binding of guanine bases (14,15).

carboxylate side chains of Glu 2 and Asp 9 were on the same side of the helical cylinder and could form 2 energetically favourable hydrogen bonds each with the bases of 2 adjacent G nucleotides as proposed by Hélène (14,15). In this context it is noticeable that in the six 17-base pair repressor binding sites of the 2 λ operators the GTGGC sequence of positions 4 to 8 has been conserved and that in four of these sites the bases of the 2 adjacent G residues were protected from methylation by dimethyl sulfate in the presence of λ repressor indicating close contacts, perhaps hydrogen bonds (16). As cro protein was shown recently to bind also to the three repressor binding sites of the right operator (17), it would be interesting to know whether the methylation pattern of these sites obtained in the presence of cro protein is the same as with λ repressor. Thus, the suggested secondary structure of the 2 cro protein fragments could explain the specific and the nonspecific interactions with nucleic acids. Nothing can be said, however, about the relative positions of the 2 identical halves of the 46-residue fragment.

Affinity chromatography of the synthetic 46-residue cro protein fragment on DNA-Cellulose. The DNA-binding properties of the 46-residue peptide were investigated further by affinity chromatography on DNA-Cellulose. Calf thymus DNA-Cellulose and λ DNA-Cellulose were prepared as described by Alberts and Herrick (18) and contained approximately 0.6mg of DNA per ml of packed material. On λ DNA-Cellulose the 46-residue peptide was resolved into 3 fractions using an NaCl gradient (10mM Tris, 1mM EDTA, 0.05M to 1M NaCl, pH 7.4). 79% of the product recovered was eluted by 0.04 to 0.09M NaCl, 16% by 0.11 to 0.17M NaCl, and 5% by 0.24 to 0.36M NaCl. These 3 fractions were most likely conformational isomers of the 46-residue peptide as they were indistinguishable with respect to amino acid composition and electrophoretic mobility. Nearly all of the 46-residue peptide applied to a calf thymus DNA-Cellulose column was eluted by 0.04 to 0.10M NaCl.

Filter binding study of the interaction of 22- and 46-residue cro protein fragment with an Eco RI digest of λ DNA. Since nitrocellulose filters retained both the 22- and the 46-residue cro protein fragment, the filter binding test (19) was used to study qualitat-

ively the interaction with an Eco RI digest of λ DNA. The incubations of the λ DNA digest with the 2 synthetic cro protein fragments were performed at pH 7.5 at 2 different salt concentrations (0.11M and 0.01M, respectively). The DNA concentration was kept constant at 10.8nM. After filtration of the incubation mixtures, the polypeptide-DNA complexes retained on the filters were analyzed by agarose gel electrophoresis at pH 8.25 and staining with ethidium bromide. At an NaCl concentration of 0.11M binding between the λ DNA fragments and the synthetic peptides was weak and there was little if any specificity of binding. At a concentration of 0.01M NaCl in the incubation mixtures, however, a preferred interaction of the 22- and the 46-residue peptide with the immunity region of λ DNA could be demonstrated. The Eco RI fragment that contains the immunity region comprises approximately 15% of the phage genome (20). In the presence of the 2 synthetic cro protein fragments at concentrations ranging from 0.024 to 1.4μM, the amount of this Eco RI fragment retained on the filter was 60-80% of the total amount of bound DNA. The remainder was mainly a fragment comprising approximately 12% of the phage genome.

Effect of the synthetic cro protein fragments on the melting point of nonhomologous DNA. In the presence of a 28μM solution of 46-residue fragment the melting point of calf thymus DNA was increased from 66.5° to 69°C whereas a 56μM solution of 22-residue fragment had almost no effect. The DNA concentration was 5.6μg per ml of 10mM Tris, 1mM EDTA, pH 7.5.

S_1-nuclease treatment of λ DNA in the presence of 22- and 46-residue cro protein fragment. Following the procedure of Vogt (21), the influence of the 2 cro protein fragments on the susceptibility of λ DNA to cleavage by single-strand specific S_1-nuclease was investigated. The results obtained are shown in Table 1. As both 46- and 22-residue cro protein fragment were capable of interacting with double-stranded and single-stranded DNA, binding of the 2 peptides may have stabilized the double-helical structure of λ DNA and may have protected transiently single-stranded segments against degradation by S_1-nuclease.

The experiments described showed that the dimeric fragment had higher affinity for nucleic acids than the monomeric fragment.

Table 1: S_1-nuclease treatment of bacteriophage λ DNA in the presence of 22- and 46-residue cro protein fragment.

	TCA-soluble λ DNA µg	%
λ DNA alone	0.27	0.9
λ DNA + S_1-nuclease	1.60	5.3
λ DNA + S_1-nuclease + 22-residue peptide	0.93	3.1
λ DNA + S_1-nuclease + 46-residue peptide	0.0	0.0

The amount of λ DNA and S_1-nuclease used per experiment was constant (30µg and 0.8µg, respectively). The concentrations of 22- and 46-residue peptide were 0.16mM and 0.07mM, respectively. Samples were incubated 2h at 37°C in S_1-nuclease buffer (2l) and then chilled. Undigested DNA was precipitated by an equal volume (i.e. 250µl) of chilled 2N TCA and centrifuged. To 0.25-ml aliquots of the supernatants 0.75ml of buffer was added and the optical absorption of the mixtures was measured spectrophotometrically at 260nm against blanks that lacked DNA. Increasing the amount of 46-residue peptide to 0.14mM in both the sample and the blank did not change the absorption difference. 66.7µg of λ DNA gave an absorbance of 1.0.

Since the 22-residue peptide formed aggregates in solvent mixtures of reduced polarity, the results obtained with the 46-residue peptide suggested that cro protein functions as an oligomer. UV difference spectroscopy and affinity chromatography demonstrated that monomeric and dimeric fragment interacted with λ DNA more strongly than with calf thymus DNA. This may be partially explained by the specific interaction of the 2 synthetic peptides with the cro protein binding site(s) of λ DNA as shown by filter binding studies using an Eco RI digest. These studies confirmed the earlier conclusion that activity as well as specificity of cro protein binding may reside in the NH_2-terminal 22-residue portion of the molecule. They also indicated that the folding of the 2 synthetic NH_2-terminal fragments may be very similar to that of the NH_2-terminus of cro protein. Monomeric and dimeric fragment showed also specificity for binding to poly(G).

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