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Involvement of outside DNA sequences in the major kinetic path by which *Eco*RI endonuclease locates and leaves its recognition sequence

(DNA–protein interactions/restriction endonuclease/facilitated diffusion mechanism)

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ABSTRACT **We have examined the kinetics of the interaction between endodeoxyribonuclease *Eco*RI (EC 3.1.23.13) and nine linear DNA fragments that range in size between 34 and 6,200 base pairs and contain the *Eco*RI site of plasmid pBR322 in a central location. The kinetic parameters governing both formation and decay of specific endonuclease·DNA complexes increase 8-fold with increasing chain length over this size range. In contrast, equilibrium competition experiments demonstrated that the intrinsic affinity of endonuclease for its recognition sequence is independent of DNA chain length over this range. Thus, DNA sequences outside the recognition site enhance the rate at which *Eco*RI endonuclease locates or leaves its recognition site without affecting the intrinsic thermodynamic parameters of site-specific interaction. These results are consistent with a facilitated diffusion mechanism for specific DNA site location by this enzyme.**

DNA recognition sequences of site-specific proteins are embedded in a large background of nonspecific DNA. Nevertheless, such proteins locate their recognition sites by kinetically efficient processes, with reported apparent second-order association rate constants approaching or exceeding those expected for diffusion-limited collision of macromolecules (1). These surprising findings led to the suggestion that the effective target size for specific DNA–protein interaction might be much larger than the size of the recognition sequence (1, 2). In such models the protein is envisioned to interact with both specific and nonspecific DNA sequences, with interactions of the latter type being on the major kinetic path by which the protein locates its recognition site. Thus, random collisions between protein and DNA would initially favor formation of nonspecific complexes, which could then be converted to site-specific complexes by a facilitated diffusion mechanism in which the protein might, for example, "slide" along the polynucleotide via a random walk until the recognition sequence is encountered.

Although theoretical treatments (2–4) have indicated that such a mechanism can account for the kinetic efficiency of site-specific proteins, direct experimental support has been limited (1, 5–8). A minimal test of this type of mechanism requires proof of interaction of the protein in question with nonspecific sequences and demonstration that such interactions are involved in the path by which the protein locates and leaves its recognition site. In this paper we demonstrate that DNA sequences external to the recognition site markedly enhance the rate at which endodeoxyribonuclease *Eco*RI (EC 3.1.23.13) locates and leaves its recognition site. The external sequences, however, are without effect on the intrinsic equilibrium constant governing specific interactions. These findings are in accord with a facilitated diffusion mechanism for specific DNA site location by this enzyme. A preliminary account of this work has been presented (5).

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MATERIALS AND METHODS

Enzymes. *Eco*RI endonuclease was the homogeneous preparation described previously (9). Endonucleases *Bsp* I (10), *Alu* I (11), and *Dpn* I (12) were purified according to published procedures, and *Bgl* II, *Pvu* II, and *Hin*fI were from commercial sources. *Bal* 31 exonuclease and polynucleotide kinase were the generous gifts of D. Robberson (University of Houston) and R. Kolodner (Harvard University), respectively.

DNA. Covalently closed pBR322 plasmid DNA (13) was isolated according to Hardies and Wells (14) and hydrolyzed with restriction endonucleases, and fragments containing the single *Eco*RI site were isolated by chromatography on RPC-5 (ref. 15; RPC-5 was the gift of G. D. Novelli, Oak Ridge Laboratories). Different restriction endonucleases yielded the following DNA fragment sizes: 4,361 base pairs (bp) (*Pvu* II, 2,067), 1,631 bp (*Hin*fI, 631), 665 bp (*Dpn* I, 315), and 34 bp (*Bsp* I and *Alu* I, 16), in which the number in parentheses is the distance in bp between the dyad of the *Eco*RI site and the nearest end. Intermediate DNA lengths (averaging 3,300, 2,500, 1,100, and 300 bp) were prepared by limited *Bal* 31 exonuclease hydrolysis (16) of the above fragments. A longer DNA fragment, 6,200 bp, was prepared by *Bgl* II cleavage of pVB301 (gift of V. Burdett, Duke University), a plasmid constructed by insertion of a 2,000-bp segment of the *Escherichia coli trp* operon (17) into the *Pvu* II site of pBR322. Hydrolysis of pVB301 with *Bgl* II yields 161-bp and 6,200-bp products. Although the orientation of the insert within pVB301 is not known, locations of the two *Bgl* II sites within the *trp* insert (17) place the single *Eco*RI site of the plasmid within 270 bp of the center of large *Bgl* II product.

DNA fragments were 5′-end labeled by using polynucleotide kinase (18) and [γ-^{32}P]ATP (>2,000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) (19).

Assays of Specific Endonuclease·DNA Complexes. Two methods were used to monitor site-specific complexes between *Eco*RI endonuclease and DNA. The first relies on retention by nitrocellulose membranes of specific endonuclease·DNA complexes formed in the absence of Mg^{2+} (9, 20, 21). Unless specified otherwise, reaction mixtures (containing 0.1 M Tris·HCl at pH 7.6, 1 mM EDTA, bovine serum albumin at 0.05 mg/ml, and end-labeled [^{32}P]DNA) were prewarmed to 37°C, *Eco*RI endonuclease was added, and incubation was continued until equilibrium was achieved. Dissociation rate constants for specific complexes (k_d) were determined by addition of 20- to 40-fold molar excess of unlabeled pBR322 DNA to equilibrium

Abbreviation: bp, base pair(s).

reactions and removal of samples (0.5 ml) as a function of time for filtration (3 ml/min) on nitrocellulose membranes (Schleicher & Schuell BA-85, 0.45-μm pore, 13-mm diameter, presoaked in 0.1 M Tris·HCl, pH 7.6/1 mM EDTA). DNA retained on filters was quantitated by liquid scintillation counting. In the case of competitive binding reactions with two DNAs of differing length, each DNA was quantitated after elution from filters (22) by electrophoresis on 12% polyacrylamide gels (23) or 1.0–1.4% agarose gels (24). Radioactive bands were excised and ^{32}P was quantitated by liquid scintillation counting.

The alternative assay is based on preferential cleavage, upon addition of Mg^{2+}, of complexes formed in the absence of a divalent cation. Site-specific complexes were formed as above, and unlabeled pBR322 was added to yield a 40-fold excess over labeled DNA. At indicated times samples were removed, $MgCl_2$ was added to 10 mM, and incubation was continued for 30 s at 37°C. Reactions were terminated by addition of an equal volume of a suspension of buffer-saturated, neutralized phenol, 2% (wt/vol) $NaDodSO_4$, and 30 mM EDTA. After extraction the phenol phase was removed and cleavage products were separated and quantitated by electrophoresis as described above.

RESULTS

Facilitated diffusion mechanisms for specific DNA site location by a protein invoke nonspecific protein–DNA interactions on the major kinetic path by which the protein locates its recognition sequence. If such nonspecific interactions are of significance, then removal of sequences flanking a recognition site would be expected to alter kinetics of formation and dissociation of specific complexes. Indeed, theoretical analyses of such mechanisms predict dependence of kinetic parameters on DNA size (2–4). While emphasis and details differ, all theoretical models predict rate enhancement with increasing DNA size up to moderately long chain lengths.

In order to assess effects of DNA size on kinetics of interaction between *Eco*RI endonuclease and its recognition sequence, we have exploited the observation that in the absence of Mg^{2+} the enzyme forms site-specific complexes with DNA that are retained with high efficiency on nitrocellulose membranes (9, 20, 21). Fig. 1 presents an analysis of dissociation rates of specific complexes between endonuclease and nine DNAs ranging in size between 34 and 6,200 bp, all of which contain the *Eco*RI site of plasmid pBR322 in a centrally located position. At the ionic strength employed (0.073 M), the lifetime of specific complexes decreases markedly with increasing DNA size in the range of 34 bp ($t_{1/2}$ = 140 min) to about 4,000 bp ($t_{1/2}$ = 15 min).* However, any increase in DNA length beyond this size did not result in further increases in the dissociation rate constant. Thus, sequences external to the *Eco*RI site enhance the rate of dissociation of specific endonuclease·DNA complexes, but there is a limit to the range of these effects.

In contrast to effects of DNA chain length on kinetics of dissociation of specific complexes, analysis of equilibrium binding suggested that the equilibrium affinity of the endonuclease for its recognition site is independent of DNA size (not shown). Competition of two DNA fragments for limiting enzyme was employed to test this possibility more carefully and to determine if association rates are also enhanced by sequences external to the *Eco*RI site. In these experiments pairwise combinations of DNA chains of differing size were mixed in 1:1 molar ratio, limiting *Eco*RI endonuclease was added, and specific complexes between endonuclease and the two DNAs were quantitated as a function of time. Fig. 2 presents results of such an experiment with 4,361- and 34-bp molecules. As is evident, both DNAs were equally represented in the final equilibrium state, and comparable results were obtained with other pairwise combinations of DNA sizes (Table 1). These findings therefore demonstrate that the intrinsic equilibrium affinity of the endonuclease for its recognition site is independent of DNA chain length.

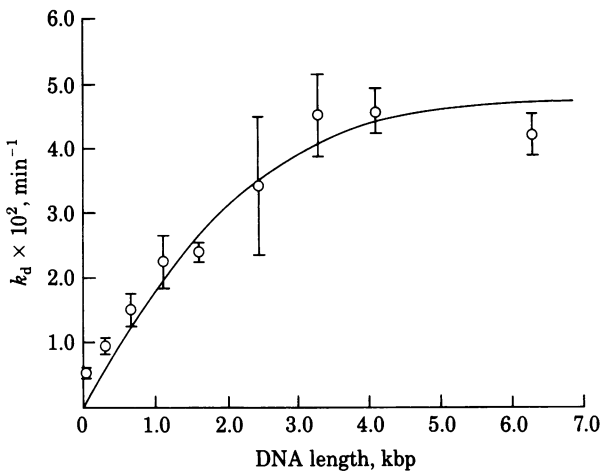

FIG. 1. Dependence of dissociation rate constant on DNA chain length. Dissociation rate constants were determined as described in the text. Rate constants were independent of concentration of nonradioactive competitor DNA over an 8-fold range and independent of initial substrate concentration in the range of 0.01–1 nM. Endonuclease was in all cases less than 2-fold molar excess (as dimer) over *Eco*RI sites, and in most cases less than equimolar. Error bars indicate 95% confidence intervals. The solid curve is a Marquardt nonlinear least-squares regression fit (25) of experimental points to the theoretical expression of Berg *et al.* (4) for a sliding mechanism:

$$k_d = \frac{k_{ass}}{K_{RO}(\Lambda l^2/4D_1)^{1/2}\coth[M(\Lambda l^2/4D_1)^{1/2}] + K_{RD}}.$$

In this formulation k_d = total dissociation rate constant from a specific site; k_{ass} = intradomain nonspecific association rate constant; Λ = intradomain nonspecific dissociation rate constant; l = length of a base pair; M = number of base pairs per chain; D_1 = diffusion constant for one-dimensional motion of protein along DNA helix; K_{RO} = equilibrium constant for specific association; and K_{RD} = equilibrium constant for nonspecific association. (For detailed discussion of these parameters, the reader is referred to ref. 4.) For *Eco*RI endonuclease and pBR322 DNA under conditions utilized here, $K_{RO} = 1 \times 10^{11}$ M^{-1} and $K_{RD} = 1 \times 10^6$ M^{-1} (unpublished data). Best fit was obtained for values of $k_{ass} = 3.1 \times 10^4$ M^{-1} s^{-1} and $(D_1/\Lambda l^2)^{1/2} = 1.3 \times 10^3$ bp.

The finding that the rate constant governing dissociation of specific complexes is dependent on DNA size whereas equilibrium stability is not would imply that DNA chain length effects should also be apparent in the association rate governing specific binding. The preferential binding of endonuclease to the longer DNA during early stages of competition experiments (Fig. 2) is in accord with this view because the observed preference must be kinetic in nature. Because initial sampling times in competition experiments were short (<0.7 min) relative to half-lives of specific complexes (>15 min), and because these experiments were performed so that less than 30% of a given DNA was involved in specific complex formation at the first sampling time, the initial preference provides a good approximation of the relative association rate of endonuclease to the two DNAs. As shown in Table 1, relative association rates estimated in this way are in good agreement with relative dissociation rates calculated from the data of Fig. 1, thus demonstrating internal consistency of kinetic and equilibrium data. These findings are

* Control experiments demonstrated that half-lives observed reflect dissociation of specific complexes rather than loss of binding activity due to enzyme inactivation.

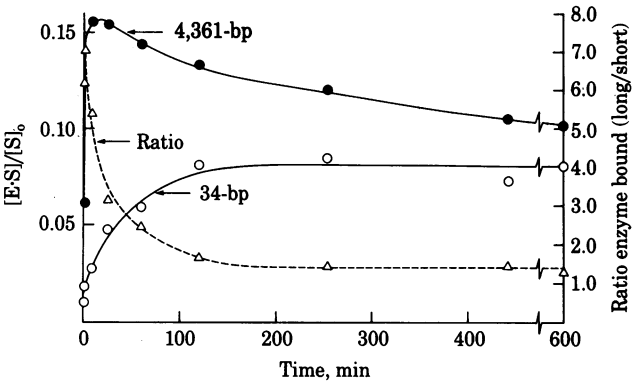

FIG. 2. Competition of 4,361- and 34-bp DNAs for binding to *Eco*RI endonuclease. Endonuclease·DNA complexes were scored by the preferential cleavage assay. Reaction mixtures (1.4 ml) containing 1 nM each ^{32}P-end-labeled 4,361-bp and 34-bp linear molecules were prewarmed to 37°C, and *Eco*RI endonuclease was added to 0.2 nM. At indicated times, samples (30 μl) were removed and supplemented with unlabeled pBR322 DNA and $MgCl_2$ to yield final concentrations of 68 nM and 9 mM, respectively. After a 30-s incubation at 37°C, cleavage reactions were terminated and quantitated. Fraction of each DNA in the enzyme-bound form ([E·S]/[S]$_0$) is shown (●, 4,361-bp DNA; ○, 34-bp DNA), as is the ratio of long DNA to short DNA in the enzyme-bound form (△).

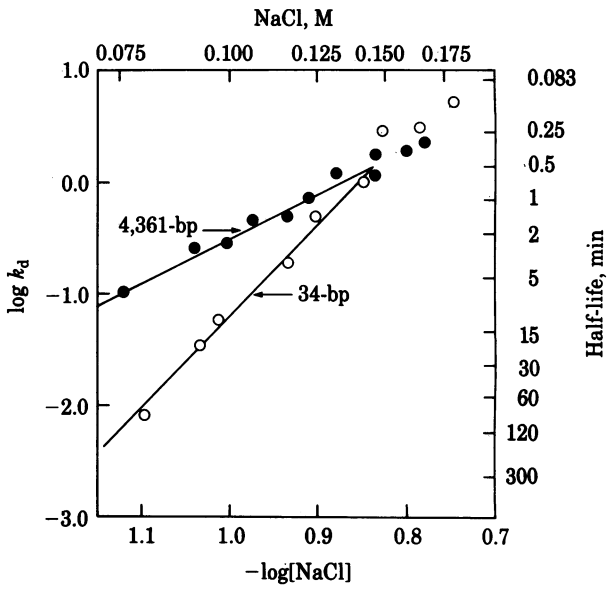

FIG. 3. Dependence of dissociation rate constants on NaCl concentration. Dissociation rate constants were determined as described in *Materials and Methods* and the legend to Fig. 1, except reaction mixtures contained 0.02 M Tris·HCl (pH 7.6), 1 mM EDTA, bovine serum albumin at 0.05 mg/ml, and NaCl as indicated. ●, 4,361-bp full-length linear pBR322; ○, 34-bp DNA. Lines represent linear least-squares regression fit of the data.

not an artifact of the nitrocellulose membrane assay because comparable kinetic and equilibrium effects were observed with the DNA cleavage assay (Table 1, Fig. 2).

Although results summarized above indicate that DNA external to the *Eco*RI site is involved in the kinetic path by which the endonuclease locates and leaves its site, they do not indicate the nature of this involvement. Facilitated diffusion-rate enhancement theories invoke nonspecific DNA–protein interactions in the process of specific site location (2–4), and previous studies have demonstrated nonspecific interaction between *Eco*RI endonuclease and a variety of polynucleotides (20, 21, 26). Because the free energy of nonspecific interactions is generally accorded a large electrostatic component, it has been argued that rate enhancement due to facilitated diffusion will be highly sensitive to ionic environment (4, 27). In particular, reduction in the affinity of a protein for nonspecific sequences with increasing ionic strength is expected to reduce the relative rate enhancement observed with increasing DNA chain length. As shown in Fig. 3, the difference between dissociation rate constants for DNAs of differing size decreases at high salt concentration, converging to a common value at a NaCl concentration of about 0.15 M (total ionic strength = 0.17 M).† This behavior is consistent with the view that length dependence of kinetic parameters is the result of reduction in nonspecific affinity at high ionic strength. However, effects of ionic strength on enzyme or DNA structure cannot be excluded.

Table 1. Relative equilibrium constants and association–dissociation rate constants for DNA pairs of differing length*

Long DNA, bp	Short DNA, bp	Relative equilibrium affinity†	Relative association rate‡	Relative dissociation rate§
6,200	34	0.90	6.1	8.2 ± 3.0
4,361	34	1.2 (1.3)	7.3 (7.0)	8.9 ± 1.5 (7.9)
4,361	665	1.0	2.0	3.1 ± 0.6
4,361	1,631	0.95	1.4	1.9 ± 0.2
1,631	34	0.88	4.9	4.6 ± 0.8
1,631	665	1.2	1.8	1.6 ± 0.3
665	34	0.97	2.8	2.9 ± 0.7

* Relative values are expressed as a ratio of that for the longer DNA to that for the shorter molecule. All values shown were determined by using the nitrocellulose membrane assay except those in parentheses, which were determined by the DNA cleavage assay.
† Determined from competition curves after achievement of equilibrium.
‡ Estimated from competition experiments (Fig. 2) as the molar ratio of endonuclease-bound DNA fragments 0.5–0.7 min after enzyme addition. In all cases, 30% or less of the longer DNA was enzyme bound at this time. In cases in which the fraction of long DNA bound approached this value, relative association rates may be underestimated by as much as 15%. This error is most significant in those experiments in which difference in chain length is largest.
§ Calculated from data of Fig. 1; ± indicates 95% confidence intervals.

In order to determine whether length effects are evident under catalytic conditions, competition cleavage experiments were performed. As shown in Table 2, the endonuclease also displays a preference for long DNA in the presence of Mg^{2+} as monitored by steady-state cleavage in reaction mixtures containing equimolar long and short molecules. This preference was evident at an ionic strength comparable to that routinely employed for filter binding assays, as well as at somewhat higher ionic strength in the buffer that has been reported to be optimal for endonuclease activity (28).

DISCUSSION

These studies indicate that DNA external to the *Eco*RI sequence is involved in the major kinetic path by which the enzyme locates and leaves its recognition site. They do not, however, explicitly identify the function of flanking sequences within the kinetic pathway. Nevertheless, the results described here are those expected for target site location by facilitated diffusion. Thus, the enhancement of kinetic parameters with increasing DNA contour length is that expected for participation of external sequences in a facilitated diffusion mechanism (2–4). Further, effects of ionic strength on the magnitude of this en-

† Relative equilibrium affinity of endonuclease for the *Eco*RI site of 4,361- and 34-bp DNAs was not altered by the increase in ionic strength.

Table 2. Kinetic preference of *Eco*RI endonuclease for long DNA under catalytic conditions

Ionic strength, M	Initial rate of cleavage, pM min^{-1}		Ratio, long/short
	34-bp	4,361-bp	
0.078	4.5	26	5.8
0.132	19	37	2.0

Reaction mixtures (37°C) contained 1 nM 34-bp and 1 nM 4,361-bp linear DNAs, 20 pM *Eco*RI endonuclease, bovine serum albumin at 0.05 mg/ml, and either (*i*) 0.085 M Tris·HCl (pH 7.6), 1 mM EDTA, 5 mM $MgCl_2$ (ionic strength 0.078 M) or (*ii*) 0.10 M Tris·HCl (pH 7.6), 0.05 M NaCl, 0.2 mM EDTA, 5 mM $MgCl_2$ (ionic strength 0.132 M). Rates shown are the averages of three determinations with independent DNA preparations.

hancement are consistent with the importance of electrostatic interactions between protein and nonspecific sequences in the process of specific site location.

Two major types of facilitated diffusion mechanisms have been considered in the literature. In the most popular model, the protein is envisioned to slide along the helix, following a random one-dimensional path, driven by thermal motion (2). We will not attempt to distinguish here such a mechanism from one in which the protein may move from one DNA site to a nearby, but nonadjacent, site via short hops due to microscopic dissociation and reassociation within the domain of the polynucleotide (4). The alternate intersegment transfer mechanism (4, 29) invokes protein transfer between distal DNA sites that may be in proximity due to flexibility of the DNA chain. Although either mechanism could yield results of the type described here, we view the latter possibility as less likely due to the rodlike behavior of short DNA chains. For example, it has been shown that while the *j* factor of linear helices varies by less than 10-fold between 4,361 and 242 bp, the *j* factor for a 126-bp chain is less than 1/100th of that for a 242-bp molecule (30). However, the dissociation rate constant of specific endonuclease·DNA complexes decreases in a simple manner at short chain lengths, and the value for a 34-bp chain is only 1/2 of that for a 300-bp molecule (Fig. 1).

The DNA length dependence observed is, however, consistent with a sliding mechanism. The curve shown in Fig. 1 represents the best fit of experimental points to the theoretical expression of Berg *et al.* (4) for the dependence of dissociation rate constant on polynucleotide contour length for a sliding mechanism. A best fit to the theoretical formulation was obtained for $k_{ass} = 3.1 \times 10^4\ M^{-1}\ s^{-1}$ and $(D_1/\Lambda l^2)^{1/2} = 1.3 \times 10^3$ bp.‡ The latter parameter is of interest because it represents the average DNA length scanned by endonuclease in the random walk process per DNA binding event under experimental conditions, if in fact a sliding mechanism is operative. It should be noted that internal consistency of parameters of the theoretical expression (Fig. 1) can be tested by virtue of the relationship $K_{RD} = k_{ass}\Lambda^{-1}$ (4). Unpublished experiments indicate a first-order rate constant for dissociation of endonuclease from nonspecific sequences in the range of 0.02–0.04 s^{-1}. This approximation for Λ and the value for k_{ass} obtained by regression analysis indicate an equilibrium constant for nonspecific binding of $0.8–1.6 \times 10^6\ M^{-1}$, in excellent agreement with the experimental value of $1 \times 10^6\ M^{-1}$ (unpublished data).‡ It can also be seen that this approximation for Λ and value of $(D_1/\Lambda l^2)$ would indicate a one-dimensional diffusion coefficient (D_1/l^2) for translation of endonuclease along the helix in the range of $10^4–10^5\ bp^2\ s^{-1}$.

The observed dependence of kinetic parameters on DNA chain length is not novel with these studies. For example, Greene *et al.* (31) have demonstrated that *Eco*RI cleavage of the duplex form of d(pTpGpApApTpTpCpA) is governed by the same k_{cat} as cleavage of simian virus 40 DNA. However, the K_m for cleavage of the octamer is 200 times that for cleavage of the natural molecule. This finding may bear on the work described here, but it is also probable that an octamer will not occupy the entirety of the DNA binding site of the endonuclease (32).

Observations similar to those made here have also been made with the extensively studied *lac* repressor–operator system by von Hippel and colleagues (8, 33). Examination of equilibrium and kinetic parameters as a function of DNA length and ionic strength has led to the conclusion that translocation along the helix by sliding is important in the mechanism of target site location by this protein, although results with this system are complicated somewhat by a marked dependence of equilibrium affinity on DNA length. The evidence in both *lac* and *Eco*RI systems suggesting involvement of facilitated diffusion in recognition site location will, we hope, encourage more direct tests of this possibility.

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‡ The value for K_{RD} used in the regression fit was determined by using a derivative of pBR322 from which the *Eco*RI site had been deleted as competitor for specific endonuclease binding to normal pBR322 or to the 34-bp fragment containing the *Eco*RI site of this plasmid. The K_{RD} value shown assumes that the competitor contains 4,400 binding sites. The validity of this assumption is not known.

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