Physical and kinetic properties of the site specific endonuclease Bam HI from *Bacillus amyloliquefaciens*

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ABSTRACT

The site specific endonuclease Bam HI which is composed of subunits of a molecular weight of 22 000 [1] can aggregate to complexes of a molecular weight of 360 000. It is an acidic protein with an isoelectric point at pH 5.3. Optimal activity is reached at 13 mM MgCl₂. A very simple method is presented to determine kinetic constants of restriction enzymes directly from agarose gel photographs without any further equipment applying the integrated Michaelis-Menten equation. With pJC 80 DNA as a substrate K_M was found to be 3.6 · 10⁻¹⁰ M. The method can be used to redefine the unit activity of site specific endonucleases unambiguously.

INTRODUCTION

Site specific endonucleases have become important tools in DNA research. Despite their widespread use only few kinetic data have been determined so that incubation times of DNA digests are often found out empirically. This might partially be due to the fact that the methods for the evaluation of K_M and V_{max} are rather tedious or require sophisticated equipment. We therefore developed a method by which the kinetic constants are directly obtained from agarose gel photographs. In this paper we present some results for the site specific endonuclease Bam HI from *Bacillus amyloliquefaciens*. In the course of this work we investigated also the dependence of the reaction rate and the state of aggregation of the enzyme on the ionic strength. Parts of the results have been presented at the NATO-EMBO Summer School on protein-nucleic acid recognition on Spetsai, August 20th to September 2nd, 1979.

MATERIALS AND METHODS

Enzymes were generally obtained from Boehringer (Mannheim), reagents were of highest purity which is commercially available.

B. amyloliquefaciens cells (RUB 500) were grown in 14 g/l Antibiotika

Bouillon (Merck, Darmstadt) at 30°C in a 500 l fermenter. Enzyme yield displayed a pronounced optimum during the log phase of growth (fig. 1). Cells were broken (500 g) by passing a 40 % suspension three times through a Manton Gaulin homogenizer. The buffer used for homogenization and also throughout the purification contained 10 mM Tris/Cl or KP_i , pH 7, 0.05 % 2-mercaptoethanol, 1 mM EDTA, 0.01 % Triton X 100, and 10 % v/v glycerol. After centrifugation any precipitate formed at 1 % polyethyleneimine concentration and 40 % ammoniumsulphate saturation was removed. Then three successive column chromatography steps were employed namely DEAE-cellulose, phosphocellulose, and hydroxyapatite. The enzyme eluted between 75 and 100 mM, 450 and 550 mM NaCl, and 120 and 150 mM KP_i resp. on these columns. After concentration by ultrafiltration the enzyme was stored at -20°C in 50 % v/v glycerol.

Enzyme activity was determined in 10 mM Tris/Cl, pH 7.3, containing 220 ng of pJC 80 DNA and 13 mM $MgCl_2$ in a total volume of 20 μ l at 37°C. The reaction was terminated and the mixture applied on 1 % agarose slab gels

Fig. 1 Bam HI activity during cell growth

Growth of *B. amyloliquefaciens* cells in a 20 l culture is measured at 578 nm (□—□). Cell bound protein is extracted by sonication and determined by the method of Lowry. It is calculated as mass of protein m_p in the entire culture (○····○). Bam activity was determined as described under METHODS. It is presented as maximal dilution for a complete digest (Δ—Δ) of 220 μ g λ -DNA in 20 μ l of the incubation mixture.

(3x170x170 mm). After electrophoresis as described by Sharp et al. [4] the DNA fragments were visualized by ethidium fluorescence and the enzyme dilution, which is sufficient for a complete reaction in 26 minutes, was determined. This corresponds to 200 U/l (see below fig 4 and RESULTS). One unit of Bam HI digests 1 pMol/min of pJC 80 DNA at V_{max}^{app} and 37°C in the above assay system.

pJC 80 DNA and λ -DNA were prepared by the method of Goebel [5] and Bøvre [6] respectively, and generously provided by H. Mayer. DNA concentrations were determined photometrically assuming that an absorbancy of 1.0 at 260 nm and 1 cm lightpath corresponds to a concentration of 50 mg/l DNA. Protein was quantitated by a modified Lowry method [7].

Sucrose gradients (4 ml, 6-30 %, in 10 mM Tris/Cl, pH 7.2, 10 mM NaCl, 1 mM EDTA, 5 % glycerol, 0.1 mM dithioerithrit, 0.1 % Triton X 100) were spun in a SW 60 rotor with catalase (beef liver, grade I), alcoholdehydrogenase (yeast), and peroxidase (horseradish, grade II) as marker proteins. Polyacrylamid gradient gels (4-26 %, 6x170x170 mm, 120 mM Tris/Cl, pH 8.0 as anode buffer, 42 mM Tris/glycin, pH 8.5 as cathode buffer) were run at 100 V with β -Lactoglobulin, ovalbumin, bovine serum albumin, and ferritin as marker proteins. SDS-polyacrylamid gels (5 % acrylamid, 5 mm wide, 70 mm long, 0.1 % SDS, 0.1 M NaP_i, pH 7.2) were electrophoresed at 5 V per gel with bovine serum albumin, ovalbumin and pepsin as marker proteins. For gelfiltration we used a Sephadex G-200 column (2.5 cm wide, 100 cm long, 10 mM KP_i, pH 7.0, 1 mM EDTA, 0.01 % β -mercaptoethanol), which was calibrated with ferritin, catalase, bovine serum albumin, and cytochrom c. Isoelectric focussing was carried out on 5 % polyacrylamid (1x100x100 mm) with an ampholyte combination in the range of pH 3.5 - 10.

THEORY

The agarose gel technique is the most common method for evaluating nuclease activities. The time t , which is necessary to achieve a complete digest of a DNA substrate, is determined. Here "complete" means that the substrate is no longer visible on the gel. During the time of the reaction the substrate concentration decreases from an initial value $[S_0]$ to a very low value $[S]$, the visibility limit. Therefore the rate is not constant during the reaction and the simple Michaelis-Menten-equation (1) can not be applied for evaluation of activities.

$$(1) \quad -\frac{d[S]}{dt} = \frac{V_{max}^{app}}{\frac{K_M^{app}}{[S]} + 1}$$

By integrating over the reaction time we get

$$(2) \quad V_{max}^{app} \cdot t = K_M^{app} \ln \frac{[S_0]}{[S]} + [S_0] - [S]$$

From this equation V_{max}^{app} can be obtained as a measure of enzyme activity. V_{max}^{app} and K_M^{app} are apparent values, because we disregard any implications of a reaction mechanism model like two step cleavage [1,2], sliding [3] etc. Equation (2) can be simplified by keeping the ratio $F = [S]/[S_0]$ constant so that we obtain

$$(3) \quad t = \frac{K_M^{app}}{V_{max}^{app}} \ln \frac{1}{F} + [S_0] \frac{1-F}{V_{max}^{app}}$$

Experimentally F is kept constant at different initial substrate concentrations by using equal amounts of substrate in every assay. Determining the visibility limit of the DNA on the gel we can calculate F . From the slope and the (extrapolated) intercept of a graph of $t = f([S_0])$ we can calculate K_M^{app} and V_{max}^{app} . $[S_0]$ must be chosen carefully as too high a value leads to long reaction times during which the enzyme activity might decrease while the lower limit is given by the inequality $[E] < [S]$, a condition for the validity of the Michaelis-Menten-equation.

We propose that the value of V_{max}^{app} be used for a new unequivocal definition of the activity unit of restriction enzymes as other methods lead to unit assignments which depend on initial substrate concentrations, either because the substrate concentrations employed fall in the range of K_M or because the extent of the reaction or the ratio F is disregarded. One unit is the amount of enzyme which at V_{max}^{app} and 37°C digests 10^{-12} M per minute of a certain substrate, preferably one which contains a single recognition site.

The temperature dependence of the Bam HI reaction can be analyzed by the same approach. Normally it is described by the proportionality

$$(4) \quad k \sim \exp - \frac{\Delta G^*}{RT}$$

where k is a rate constant, ΔG^* the activation energy of the reaction,

R the gas constant, and T the absolute temperature. According to the Michaelis-Menten theory $V_{\max}^{\text{app}}$ is directly proportional to k

$$(5) \quad V_{\max}^{\text{app}} = k [E]$$

where [E] is the enzyme concentration. So in equation (4) we can substitute k by $V_{\max}^{\text{app}}$. As $V_{\max}^{\text{app}} \sim \frac{1}{t}$ because of equation (3) we can use

$$(6) \quad k \sim V_{\max}^{\text{app}} \sim \frac{1}{t} \sim \exp - \frac{\Delta G^*}{RT}$$

or

$$(7) \quad \ln \frac{1}{t} \sim - \frac{\Delta G^*}{RT}$$

instead of equation (4). A plot of $\ln 1/t$ vs. $1/T$ can thus be used to determine ΔG^* .

RESULTS AND DISCUSSION

The results of enzyme purification are shown in tab. 1. After the phosphocellulose chromatography the preparation was completely free of non-specific nucleases. Except when otherwise indicated we used the pooled hydroxyapatite fractions for all the experiments reported here.

In 0.1 % SDS-polyacrylamid gels a single band runs at a molecular weight of 90,000, when 6 μg of protein is applied. As the visibility limit of the method is about 1 μg of protein Bam HI is approximately 85 % pure in this preparation.

On G-200 Sephadex the molecular weight of the enzyme was approximately

Tab. 1 Results of Bam HI purification

fraction	total protein mg	total activity U	specific activity U/mg	yield %
Crude extract	35 200	[In crude preparations activities cannot be determined accurately due to the presence of non-specific nucleases]		
Polyethyleneimine supernatant	19 200			
Ammoniumsulphate supernatant	12 400			
DEAE-cellulose column	1 060	7 500	7	100
Phosphocellulose column	14	2 400	170	32
Hydroxyapatite column	2	500	250	7

270,000. The subunit composition of Bam HI was determined by sucrose gradient centrifugation [8] and electrophoresis in polyacrylamid gradient gels. The gradients were fractionated or sliced and eluted. Then they were assayed for Bam activity. The results are shown in tab. 2. It can be concluded that the monomer, molecular weight 22,000 [1], can exist in highly aggregated forms. The monomer could not be detected in some of the gels, because they were run too long so that the small species had already passed it. In sucrose gradients the monomer was not found at all. It apparently does not exist in solution. When it is generated artificially as on the gels, it is still active - but not necessarily as a monomer. The relative amounts of different aggregated forms as shown in tab. 2 can not be used to calculate the subunit

Tab. 2 Different aggregated forms of Bam HI

method	no.	probe: enzyme after	molecular weight in thousands % of recovered activity						
polyacryl- amid gradients	1	phosphocellulose column	43 36	65 57	185 7				
	2	phosphocellulose column	24 62	68 18	88 18	270 2			
	3	phosphocellulose column	44 8	65 6	88 22	170 64			
	4	crude extract	44 38			187 52	278 10		
	5	phosphocellulose column	20 (not determined)	300					
sucrose	1	hydroxyapatite column	45 38	370 62					
gradient	2				80 24	180 35	265 25	380 20	
centri-	3		42 90	95 5		360 5			
fugation	4		48 77	87 23					
gelfiltration	1 + 2	DEAE cellulose column	270 -						
subunit composition theoretical molecular weight			2 44	3 66	4 88	8 176	12 264	16 352	

association constants or to identify the prominent native form of the enzyme, because neither of the separations is an equilibrium method. The relative amounts of the larger species must be underestimated. The methods were designed to simulate assay conditions with respect to low ionic strength. This explains the presence of aggregates beyond the tetramer, which was the biggest species found by Smith and Chirikjian [11]. The lowest line in tab. 2 shows that the aggregates correspond to the dimer, trimer, tetramer, octamer, dodecamer, and hexadecamer of the 22,000 molecular weight subunit.

Isoelectric focussing was carried out with an ammoniumsulphate supernatant fraction. Following electrophoresis part of the gel was stained with Coomassie Blue, part was sliced and eluted. 80 % of the recovered Bam activity was found in a single slice at pH 5.3.

The temperature dependence of the reaction was studied in the range between 4 and 56°C. Above 45°C the enzyme became gradually unspecific as indicated by slight smearing of the bands. Above 56°C it was rapidly inactivated. As inactivation is not observed below 40°C [9] we plotted the data in the range of 4 to 37°C in fig. 2. In this experiment λ -DNA was used as the substrate. The activation energy of the catalyzed cleavage was calcu-

Fig. 2 Temperature dependence of Bam activity

Activity was determined as described under METHODS but with λ -DNA instead of pSC 80. The enzyme was diluted 20fold. The logarithm of the reciprocal of time t for a complete digest of 220ng λ -DNA is shown as a function of the reciprocal of the absolute temperature T . The slope is used to determine the activation energy for the catalyzed reaction.

lated according to equation (7) as $(5.6 \pm 0.4) \cdot 10^3$ J/mol.

The influence of the cofactor concentration was investigated up to 100 mM MgCl_2 . An optimum of activity was reached at 13 mM (fig. 3). It was very broad and extended up to 17 mM (not shown). Beyond this concentration the enzyme became less effective, because of the increase of ionic strength. This was proved by experiments with other ions, namely sodium chloride and potassium phosphate, which produced the same effect as MgCl_2 : between an ionic strength of 50 and 100 mM activity and specificity dropped gradually (slight smearing), above 100 mM the enzyme was completely inhibited. So it is not sure whether 13 mM is the true optimum or whether a salt effect is superimposed. Salt effects might also be the reason for the lower optimum reported by Smith and Chirikjian [1] who used a stronger buffer for their assay. Mg^{2+} could be replaced by Mn^{2+} and Co^{2+} , but the activity and the specificity (slight smearing) were lowered. The band pattern was conserved nevertheless.

The Michaelis parameters of the Bam HI endonuclease are determined according to equation (3) with pJC80 DNA as a substrate. Every assay contained 225 ng of DNA. The visibility limit was determined as 10 ± 2 ng. The

Fig. 3 Cofactor requirement

As described under METHODS the time t necessary for complete cleavage of 220 ng λ -DNA is determined at different concentrations of MgCl_2 . The enzyme was diluted 75fold. The dashed line marks the shortest possible reaction time of 42 min.

enzyme was diluted 75fold in the assay mixture. The activity of the enzyme did not decrease during the time needed for the reaction. This was shown by adding an equal amount of substrate after the first reaction was gone to completion. The second digest was accomplished in the same time as the first. From the slope of the plot of t vs. $[S_0]$ (fig. 4) we found $V_{\max}^{\text{app}} = 1.1 \cdot 10^{-10} \text{ M min}^{-1}$. K_M^{app} was calculated from the intercept as $K_M^{\text{app}} = 3.6 \cdot 10^{-10} \text{ M}$. It is one order of magnitude lower than the value for Eco RI on ColEI at 37°C [10], which is $8 \cdot 10^{-9} \text{ M}$.

The value of $V_{\max}^{\text{app}}$ as calculated from fig. 4 can be used for the assessment of enzyme activities and the activity unit. In the past the unit of activity based on reaction rates has often been determined neglecting

Fig. 4 Kinetic constants of Bam HI

Bam HI is incubated with 225 ng of pJC 80 DNA in different volumes of the incubation mixture as described under METHODS, and the time t necessary for complete reaction is determined. The symbol for complete reaction is (▲), for incomplete (△). Any pair of coordinates above the straight line represents conditions for a complete reaction, below the line conditions do not lead to a complete digest.

its relation to $V_{\max}$ (equation (5)). Thus the unit itself became dependant on substrate concentrations and was often not linearly dependant on $[E]$. As K_M values were generally not known the units were not comparable. Furthermore, predictions for concentrations and reaction times of DNA digests could not be made with these data, but had to be found out empirically.

One new unit is the amount of enzyme which cleaves 10^{-12} M pJC80 DNA per minute at substrate saturation - 90 % saturation is reached at 116 mg/l in our case! In fig. 4 we found $V_{\max}^{\text{app}} = 110 \cdot 10^{-12} \text{M} \cdot \text{min}^{-1}$, which means that we had 110 U/l in the assay. During the purification (see above MATERIALS and METHODS) we used a substrate concentration of 11 mg/l. From fig. 4 which is now used as a standard curve we find that we need 47.5 minutes for a "complete" digest at an enzyme concentration of 110 U/l. As $V_{\max}^{\text{app}} \sim [E] \sim 1/t$, because of equations (3) and (5), at 200 U/l we need 26 minutes.

The values of K_M^{app} and $V_{\max}^{\text{app}}$ are now only dependant on the nature of the substrate and on the reaction mechanism. With λ -DNA we would only get mean values of K_M and $V_{\max}$, because it has five sites for the enzyme which are not cleaved at equal rates (data not shown) as also reported for the reaction of EcoRI with this substrate [11]. This is probably caused by a sensitivity of the enzyme to the environment of the hexamer recognition site. That the activity of polymer degrading enzymes depend on the substrate even if identical sites are cleaved is not a unique feature of restriction enzymes but can be observed for proteases and other enzymes as well. It is therefore expected that different DNA's yield different kinetic parameters. Moreover K_M could be influenced by the mechanism, if Bam is a processive enzyme which stays on DNA after cleavage of one site to find the next. pJC80 DNA, a derivative of pBR 322, contains only one Bam site so that these problems are eliminated. The constant can still be influenced by unspecific binding as described by Goppelt et al. for the EcoRI endonuclease [12].

CONCLUSIONS

The site specific endonuclease Bam HI is an acidic protein which is composed of subunits of a molecular weight of 22,000. In solution different aggregated forms are observed, the dimer, tetramer, and octamer being the prominent species. The association is strongly favored in low salt and even in the presence of 0.1 % SDS the tetramer is not dissociated. This suggests that in the assay the highly aggregated forms prevail, because it requires an ionic strength of lower than 50 mM for optimal activity. Together with Bam HI which cleaves the hexanucleotide sequence GGATCC [13] a number of

endonucleases is known, which recognize the same central tetranucleotide, but have different specificities at the outer positions [14]. These are of interest for comparative studies of protein nucleic acid recognition. We believe that the use of the integrated Michaelis-Menten-equation as presented in this paper can serve as a fast and convenient means for kinetic studies of restriction endonucleases and their interaction with different substrates. When applied on Bam we found a K_M^{app} of $3.6 \cdot 10^{-10}$ M on pJC80 DNA. Biologically this seems reasonable for restriction as well as for recombination functions [15]. If a single virus, whose DNA has only a single Bam site, infects a bacillus cell the volume of which is $1.4 \cdot 10^{-15}$ l [16], the concentration of sites in the cell is $1.2 \cdot 10^{-9}$ M, which corresponds to 75 % saturation of enzyme. A similar calculation can be made for host DNA sites. An assessment of reaction rates in vivo is more difficult, because it is not known whether Bam is fixed in the cell membrane or whether it can diffuse freely in the cytoplasm. The in vivo concentration of Bam is probably larger than 25,000 U/l as 7,500 U were found in the DEAE-cellulose eluate of 500 g or roughly 0.3 l of cells. From this value, the volume per Bacillus cell, and the reported values for purity, yield, and subunit molecular weight of this preparation we calculate a copy number of 3400 subunits per cell during the middle of the log phase.

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