Analysis of the reaction mechanism of the non-specific endonuclease of *Serratia marcescens* using an artificial minimal substrate

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Abstract We have studied the mechanism of action of the *Serratia* nuclease using deoxythymidine 3′,5′-bis-(*p*-nitrophenyl-phosphate) as a substrate. A comparison of the activity with which the wild-type enzyme and several mutant enzymes attack this artificial substrate and herring sperm DNA, respectively, supports the suggestion that His^{89} is the general base and a Mg^{2+} ion bound to Glu^{127} the general acid in the mechanism of phosphodiester bond hydrolysis by the *Serratia* nuclease, and that Asn^{119} directly participates in catalysis, for example by transition state stabilisation. Arg^{57}, Arg^{87} and Arg^{131}, essential for nuclease activity, are not needed for cleavage of the artificial substrate, suggesting that they are involved in binding and positioning of nucleic acid substrates.

Key words: DNase I; Staphylococcal nuclease; Phosphodiesterase; Enzyme mechanism; Acid base catalysis; Deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate)

1. Introduction

Serratia marcescens secretes several hydrolases including an endonuclease [1,2] which catalyses the cleavage of ss and ds DNA and RNA in the presence of Mg^{2+} to give 5′-phosphorylated (mono-), di-, tri- and tetranucleotides [3,4]. It is a non-specific nuclease but like other non-specific endonucleases (reviewed in [5,6]) shows certain sequence preferences [7,8].

The crystal structure analysis [9,10] together with a site-directed mutagenesis study which was based on a sequence alignment of six related nucleases [11,12] revealed five conserved amino acid residues to be of particular importance for catalysis: Arg^{57}, Arg^{87}, His^{89}, Asn^{119} and Glu^{127}. Among these, H89A, N119A and E127A are the mutants with the lowest residual DNA cleavage activity, namely $<0.001\%$ (H89A, N119A) and 0.1% (E127A) of the activity of the wild-type enzyme. A detailed biochemical investigation suggested that His^{89} might be the general base, which activates the attacking nucleophile, while Glu^{127} might be involved in protonation of the leaving group and together with Asn^{119} could serve to bind the essential cofactor Mg^{2+} [12] (Fig. 1). Several positively charged amino acid residues, for example Arg^{87} and Arg^{131}, which in the crystal structure flank a cleft region near the putative active site are likely to constitute the substrate binding site [9].

In this paper we present results of cleavage experiments with deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate), a synthetic substrate which is also hydrolysed by other non-specific nucleases like staphylococcal nuclease and DNase I. This substrate is attacked on the 5′-side by staphylococcal nuclease [13] and the *Serratia* nuclease [14], and on the 3′-side by DNase I [15]. While a free *p*-nitrophenolate ion is released by DNase I and the *Serratia* nuclease, staphylococcal nuclease produces *p*-nitrophenyl phosphate (Fig. 2). In the present study this synthetic substrate has been used to examine mutant enzymes in which amino acid residues were replaced that are likely to be involved in substrate binding or catalysis of the *Serratia* nuclease. Based on the results obtained, supporting evidence for the role of various amino acid residues in the mechanism of DNA cleavage by the *Serratia* nuclease is given.

2. Materials and methods

2.1. Chemicals, enzymes and DNA

If not otherwise stated all chemicals were obtained from Merck. DNase I, shrimp alkaline phosphatase, T4 polynucleotide kinase and γ-[^{32}P]ATP were obtained from Amersham. All enzymes were used according to the respective manufacturer's recommendations. Herring sperm DNA was from Promega.

2.2. Deoxythymidine 3′,5′-bis-(p-nitrophenyl phosphate)

Deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) was a kind gift of Prof. Dr. B.A. Connolly (Newcastle). The concentration of this analogue was determined by measuring the absorbance at 275 nm using an extinction coefficient of 22 500 M^{-1} cm^{-1} [13].

2.3. Serratia nuclease and mutants

Purification of the wild-type *Serratia* nuclease and the mutant enzymes R57A, R57K, R87A, R87K, H89A, N119A, N119Q, E127A and R131A were produced as His_6-tagged proteins in *E. coli* and purified as described in detail before [11,12].

2.4. Activity measurement with DNA as substrate (hyperchromicity assay)

The DNase activity of the wild-type *Serratia* nuclease and different mutants was measured using a hyperchromicity assay with high-molecular-weight herring sperm DNA as substrate as described recently [14].

2.5. Cleavage of deoxythymidine 3′,5′-bis-(p-nitrophenyl phosphate)

To identify the products of the hydrolysis of the synthetic substrate deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) by *Serratia* nuclease 35 μM substrate was incubated with 5 μM wild-type enzyme in 50 mM Tris-HCl, pH 8.2, 5 mM $MgCl_2$ in a 1 cm cuvette at 25°C. Spectra were recorded from 250 to 450 nm with a scan time of 40 s per spectrum on a Hitachi U-3000 spectrophotometer over a time course of 160 min.

The rate of cleavage of deoxythymidine-3′,5′-bis-(*p*-nitrophenyl phosphate) by the *Serratia* nuclease and different mutants was determined via the absorbance increase at 400 nm which is typical for the cleavage product *p*-nitrophenol in its unprotonated form (ε_{400}=17 200 M^{-1} cm^{-1}) [15]. Substrate concentrations were varied between 0.1 and 34 mM. Reactions were carried out in 50 mM Tris-HCl, pH 8.2 and 5 mM $MgCl_2$, if not stated otherwise. From the linear part of the reaction progress curve rates were determined and plotted against the substrate concentration. The slope of this curve gives the k_{cat}/K_M values of the hydrolysis reactions.

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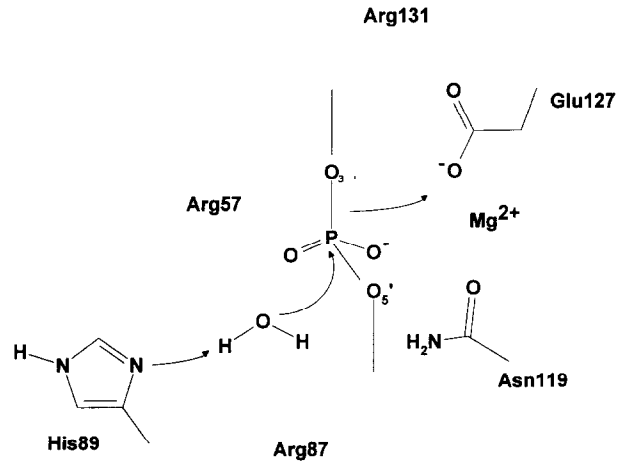

Fig. 1. A model of the active site of the *Serratia* nuclease based on the structure analysis [9] and a site-directed mutagenesis study [12].

3. Results

The hydrolysis of deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) by the *Serratia* nuclease is accompanied by a marked increase of absorption at 400 nm wavelength, indicating *p*-nitrophenol to be the cleavage product (Fig. 3). No measurable change of absorbance occurred at 330 nm, which would be characteristic for the production of *p*-nitrophenyl phosphate. Treatment of the hydrolysis products with shrimp alkaline phosphatase prior to labeling with γ-[^{32}P]ATP and T4 polynucleotide kinase shows that *Serratia* nuclease cleaves this substrate on the 5′ side, leaving deoxythymidine 3′-(*p*-nitrophenyl phosphate) still accessible for DNase I [14]. Different from DNA and RNA substrates whose cleavage by the *Serratia* nuclease is absolutely dependent on Mg^{2+} ions, deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) hydrolysis does not require Mg^{2+}. In the presence of EDTA, the wild-type enzyme cleaves deoxythymidine-3′,5′-bis-(*p*-nitrophenyl phosphate) with the same rate as in the presence of Mg^{2+} (data not shown). For the purpose of comparison, all cleavage experiments were carried out in the presence of Mg^{2+}.

As it was not possible to reach substrate saturation in the *Serratia* nuclease-catalysed cleavage of deoxythymidine-3′,5′-bis-(*p*-nitrophenyl phosphate), only the k_{cat}/K_M could be determined (Fig. 4). Its value (2.2×10^{-6} s^{-1} μM^{-1}) is about

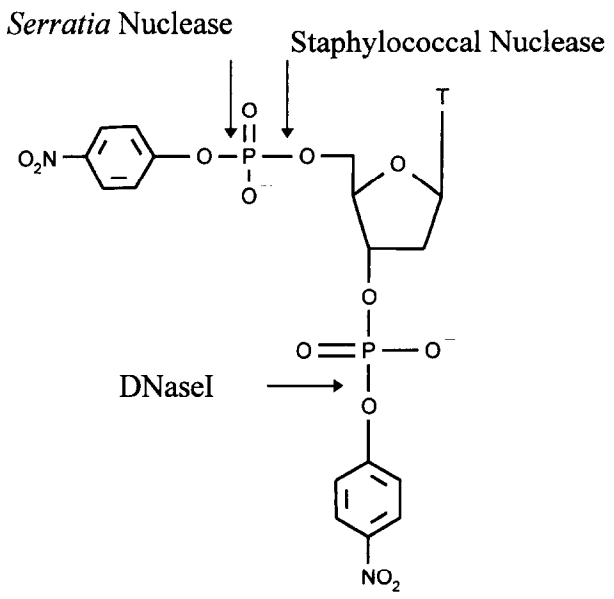

Fig. 2. Structure of deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) with the cleavage sites for the *Serratia* nuclease, staphylococcal nuclease and DNase I.

6 orders of magnitude smaller than that determined for high-molecular-weight DNA (4.7 s^{-1} μM $(nt)^{-1}$) (Table 1).

Based on a detailed mutational analysis His^{89}, Asn^{119}, Glu^{127} and Arg^{57} have been suggested previously to be essential for the catalysis of the *Serratia* nuclease [12] (Table 1). The H89A and N119A mutants possess less than 0.001% of the wild-type DNA cleavage activity; with the synthetic substrate deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) no measurable cleavage occurs (Table 1). The conservative exchange of Asn^{119} to glutamine results in an enzyme with 0.1% residual DNA cleavage activity, the activity towards deoxythymidine-3′,5′-bis-(*p*-nitrophenyl phosphate) is reduced to 15% of the wild-type activity (Table 1). In contrast, the E127A mutant has a relative activity of 0.1% towards DNA, but catalyses the cleavage of deoxythymidine-3′,5′-bis-(*p*-nitrophenyl phosphate) with essentially the same rate as the wild-type enzyme (Table 1). A similar result was obtained for the R57A and R57K mutants, whose DNA cleavage activities relative to the wild-type enzyme are 0.6% and 6%, respectively, while the activity towards the synthetic substrate

Table 1
Relative kinetic parameters for the cleavage of high-molecular-weight DNA (taken from [12]) and deoxythymidine-3′,5′-bis-(*p*-nitrophenyl phosphate) (npdTpn) by the wild-type and several mutants of the *Serratia* nuclease

Enzyme	Rel. k_{cat} (DNA)[a]	Rel. $1/K_M$ (DNA)[a]	Rel. k_{cat}/K_M (DNA)[a]	Rel. k_{cat}/K_M (npdTpn)[b]
Wild-type	1	1	1	1
R57A	0.017	0.37	0.006	2.5
R57K	0.005	12.5	0.06	1
R87A	0.34	0.009	0.003	1.5
R87K	0.14	3.8	0.5	2
H89A	n.d.	n.d.	$< 10^{-5}$	n.d.c.
N119A	n.d.	n.d.	$< 10^{-5}$	n.d.c.
N119Q	0.002	0.63	0.001	0.15
E127A	0.039	0.028	0.001	1
R131A	0.24	0.029	0.07	4

[a]For wild-type nuclease the following values were found for cleavage of herring sperm DNA with the hyperchromicity assay: k_{cat}=3400 s^{-1}, K_M=722 μM (nt), k_{cat}/K_M=4.7 s^{-1} μM $(nt)^{-1}$.
[b]For wild-type nuclease a k_{cat}/K_M of 2.2×10^{-6} s^{-1} μM^{-1} was determined for cleavage of deoxythymidine-3′,5′-bis-(*p*-nitrophenyl phosphate).
n.d.: not determined; n.d.c.: no detectable cleavage.

deoxythymidine-3′,5′-bis-(*p*-nitrophenyl phosphate) is not affected by mutations at this position (Table 1).

Different from the mutants just described, mutants at position Arg^{87} and Arg^{131} are only slightly affected in their k_{cat} with respect to DNA cleavage activity. This is the reason why these arginine residues are thought to be involved in substrate binding and not directly involved in catalysis. R87A and R131A, which have residual DNA cleavage activities of 0.3 and 7%, respectively, catalyse the cleavage of deoxythymidine-3′,5′-bis-(*p*-nitrophenyl phosphate) 1.5 and 4 times better than the wild-type nuclease. The substitution of Arg^{87} by lysine results in a mutant with half the wild-type DNA cleavage activity and 2-fold higher activity towards deoxythymidine-3′,5′-bis-(*p*-nitrophenyl phosphate) (Table 1).

4. Discussion

Deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) is cleaved by different non-specific endonucleases like staphylococcal nuclease and DNase I and also functions as a substrate for the *Serratia* nuclease. Deoxythymidine 5′-(*p*-nitrophenyl phosphate) is not cleaved by the *Serratia* nuclease (Kolmes, unpublished), probably because of the absence of a 3′-phosphate, as recent experiments with short oligonucleotides had shown that a phosphate 3′ next to the scissile phosphodiester bond is essential for cleavage [14]. Deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate), therefore, can be considered to be a minimal substrate for the *Serratia* nuclease with only one defined cleavage site. As such it can be used to analyse the integrity of the catalytic center of mutants of the *Serratia* nuclease, in a similar manner as reported recently for DNase I [16]. It can also be used to find out which amino acid residue functions as the general acid involved in leaving group protonation as demonstrated for RNase A with uridine 3′-(*p*-nitrophenyl phosphate) [17].

In this work we have examined the activity of several mutants of the *Serratia* nuclease using deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) as the substrate to get further insight into the function of amino acid residues suggested to be important for catalysis of phosphodiester bond hydrolysis by the *Serratia* nuclease.

The results of mutagenesis experiments and the analysis of the pH dependence had suggested that the conserved His^{89} which is essential for the catalysis of the *Serratia* nuclease is

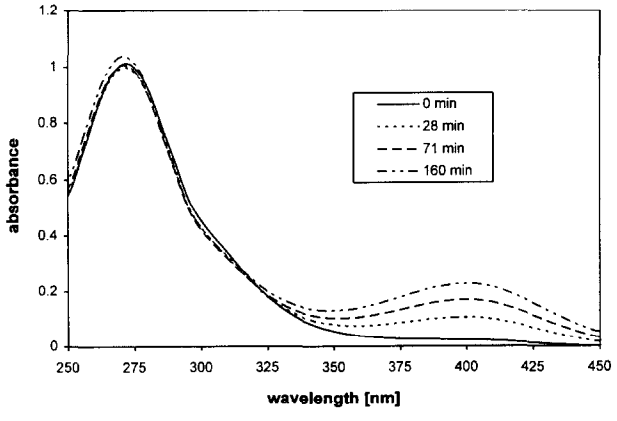

Fig. 3. Kinetics of cleavage of 35 μM deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) by 5 μM *Serratia* nuclease monitored by the change in absorbance between 250 and 450 nm over 160 min.

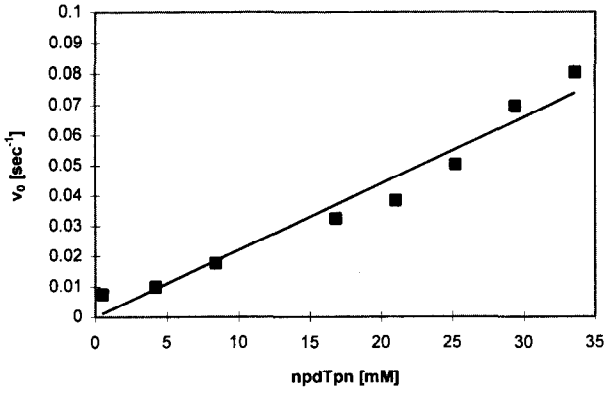

Fig. 4. v_0/[S]-diagram of the cleavage of deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) by wild-type *Serratia* nuclease. Initial rates were determined by measuring the increase in absorbance at 400 nm caused by the release of *p*-nitrophenol.

likely to act as the general base [12]. The results reported here with the synthetic substrate deoxythymidine-3′,5′-bis-(*p*-nitrophenyl phosphate), which is not cleaved by a mutant in which this amino acid was replaced by alanine, support the importance of this residues for the catalytic activity of the *Serratia* nuclease. Asn^{119} has been implicated in transition state stabilisation, for example by positioning of the attacking water molecule. The inability of the N119A mutant to cleave deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) and the reduced activity of the N119Q mutant is in agreement with this vital function.

A striking result of the present study concerns the cleavage of the substrate deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) by E127A. Although Glu^{127} is catalytically important as demonstrated by the fact that the activity of the alanine mutant towards high molecular weight DNA is reduced to 0.1% of the wild-type activity, cleavage of deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) is not at all affected by this mutation. An explanation for this could be the low pK_a of 7.14 of the conjugate acid of the *p*-nitrophenolate leaving group [18], which is much smaller than the pK_a of ~16 of the leaving group of DNA or RNA substrates [19]. Therefore, protonation of the leaving group is not necessary when deoxythymidine-3′,5′-*p*-(nitrophenylphosphate) is the substrate, but essential for nucleic acid substrates. We had proposed previously [12] that Glu^{127} could be indirectly involved in leaving group stabilisation by binding a Mg^{2+} ion which could associate itself with the leaving group or that leaving group protonation could occur at the expense of a Mg^{2+}-bound water molecule. The absence of an effect of the $Glu^{127} \rightarrow Ala$ substitution on cleavage of deoxythymidine-3′,5′-bis-(*p*-nitrophenyl phosphate) and the fact that neither the wild-type enzyme nor the E127A mutant require Mg^{2+} for cleavage of this artificial substrate suggests that Mg^{2+} bound to Glu^{127} could indeed be the general acid which protonates the leaving group during DNA and RNA cleavage by the *Serratia* nuclease. Using the same approach it was demonstrated that in RNase A His^{119} functions as the general acid because the H119A mutant was able to cleave uridine 3′-(*p*-nitrophenyl phosphate), not, however, uridylyl(3′→5′)-adenosine or polyC [17]. Our results imply that Glu^{127} cannot be the general base rather than His^{89} as discussed as an alternative recently [9]. By the same argument His^{89} cannot be the general acid in the mechanism of cleavage of nucleic acids by the

Serratia nuclease, because in this case the H89A mutant should be able to cleave deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) which does not require protonation of its leaving group.

Arg^{57} is a catalytically important amino acid because substitutions by alanine or even lysine lead to mutants with very low activity towards high-molecular-weight DNA, mainly due to a low k_{cat}, R57A and R57K, however, exhibit the same activity as the wild-type enzyme towards deoxythymidine-3′,5′-bis-(*p*-nitrophenyl phosphate). Arg^{57} is thought to be involved in transition state stabilisation [12]; this could include a distortion of the nucleic acid. For the very short substrate deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) such a distortion might not be necessary, such that mutations at this position do not have any effect on the cleavage of deoxythymidine-3′,5′-bis-(*p*-nitrophenyl phosphate). Our results exclude that Arg^{57} is involved in transition state stabilisation by neutralising the extra negative charge which transiently forms when a hydroxide ion attacks the phosphorous of the scissile phosphodiester bond, because also with deoxythymidine-3′,5′-bis-(*p*-nitrophenyl phosphate) as substrate a pentacovalent phosphorous is likely to be formed.

Arg^{87} and Arg^{131} are two out of at least 10 basic amino acid residues that are located within the presumptive substrate binding site which is formed as a deep cleft on the surface of the *Serratia* nuclease close to the essential active-site His^{89} [9]. The fact that mutants of these basic residues, which are mainly affected in their K_M for DNA, cleave the synthetic substrate deoxythymidine-3′,5′-bis-(*p*-nitrophenyl phosphate) with wild-type activity, support the suggestion that these amino acid residues are involved in substrate binding rather than in catalysis. Deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) as a minimal substrate for the nuclease presumably is too short to be affected by changes at these residues. Previous experiments with short oligo dA substrates had shown that Arg^{131} is likely to be involved in binding the phosphate 5′ to the scissile phosphodiester bond, while Arg^{87} seems to be associated to the phosphate group next to the 3′-phosphate [20]. These contacts are not necessary for binding deoxythymidine-3′,5′-bis-(*p*-nitrophenyl phosphate) such that exchanges of Arg^{87} and Arg^{131} have no influence on cleavage of this substrate.

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References

[1] Eaves, G.N. and Jeffries, C.D. (1963) J. Bacteriol. 85, 273–278.
[2] Ball, T.K., Saurugger, P.N. and Benedik, M.J. (1987) Gene 57, 183–192.
[3] Nestle, M. and Roberts, W.K. (1969) J. Biol. Chem. 244, 5213–5218.
[4] Nestle, M. and Roberts, W.K. (1969) J. Biol. Chem. 244, 5219–5225.
[5] Saenger, W. (1991) Curr. Opin. Struct. Biol. 1, 130–138.
[6] Suck, D. (1992) Curr. Opin. Struct. Biol. 2, 84–92.
[7] Yonemura, K., Matsumoto, K. and Maeda, H. (1983) J. Biochem. Tokyo 93, 1287–1295.
[8] Meiss, G., Friedhoff, P., Gimadutdinow, O., Hahn, M. and Pingoud, A. (1995) Biochemistry 34, 11979–11988.
[9] Miller, M.D., Tanner, J., Alpaugh, M., Benedik, M.J. and Krause, K.L. (1994) Nature Struct. Biol. 1, 461–468.
[10] Miller M.D. and Krause, K.L. (1996) Protein Sci. 5, 24–33.
[11] Friedhoff, P., Gimadutdinow, O. and Pingoud, A. (1994) Nucleic Acids Res. 22, 3280–3287.
[12] Friedhoff, P., Kolmes, B., Gimadutdinow, O., Wende, W., Krause, K.L. and Pingoud, A. (1996) Nucleic Acids Res. 24, 2632–2639.
[13] Cuatrecasas, P., Wilchek, M. and Anfinsen, C.B. (1969) Biochemistry 8, 2277–2284.
[14] Friedhoff, P., Meiss, G., Kolmes, B., Pieper, U., Gimadutdinow, O., Urbanke, C. and Pingoud, A. (1996) Eur. J. Biochem. (in press).
[15] Liao, T. (1975) J. Biol. Chem. 250, 3721–3724.
[16] Doherty, A.J., Worrall, A.F. and Connolly, B.A. (1995) J. Mol. Biol. 251, 366–377.
[17] Thompson, J.E. and Raines, R.T. (1994) J. Am. Chem. Soc. 116, 5467–5468.
[18] Fickling, M.M., Fischer, A., Mann, B.R., Packer, J. and Vaughan, J. (1959) J. Am. Chem. Soc. 81, 4226–4230.
[19] Gerlt, J.A. (1963) in: Nucleases (Linn, S.M., Lloyd, R.S. and Roberts, R.J., Eds.), pp. 1–34. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[20] Friedhoff, P. (1995) Doctoral Thesis, University of Giessen.