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5-Methyldeoxycytidine monophosphate deaminase and 5-methylcytidyl-DNA deaminase activities are present in human mature sperm cells

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Abstract Human mature sperm cells have a high nuclease and 5-methyldeoxycytidine monophosphate (5-mdCMP) deaminase activity. The deaminase converts the nuclease degradation product 5-mdCMP into dTMP which is further cleaved into thymine and the abasic sugar-phosphate. Both 5-methylcytidine 5′ and 3′ monophosphates are good substrates for the deaminase. 5-methylcytidine is not a good deaminase substrate and 5-methylcytosine (5mC) is not a substrate. A purified fraction of the deaminase free of nucleases deaminates 5mC present in intact methylated double-stranded DNA. 5-mdCMP deaminase co-purifies on SDS–PAGE with dCMP deaminase and has an apparent molecular weight of 25 kDa. The enzyme requires no divalent cations and has a K_m of 1.4×10^{-7} M for 5-mdCMP and a V_{max} of 7×10^{-11} mol/h/μg protein. The possible biological implications of the deaminase's activities in the present system are discussed.

Key words: Paternal genome; Mutations; DNA degradation; DNA deamination; DNA demethylation

1. Introduction

Gene silencing could possibly occur through enzymatic methylation of DNA ([1,2] and references therein) and/or the incorporation of 5-methyldeoxycytidine monophosphate (5-mdCMP) into DNA which is undergoing repair [3,4,5]. Holliday and Ho [4,5] have isolated a strain of Chinese hamster ovary (CHO) cells which spontaneously silences two selected genes at high frequency. This strain of CHO cells had a very low 5-mdCMP deaminase activity and efficiently incorporated 5-mdCMP into DNA. In contrast, another strain of CHO cells with high deaminase activity had a very low frequency of gene silencing [4,5]. It has also been shown that under special conditions deamination can occur on methylated Cs in DNA. For example, in the absence of the cofactor *S*-adenosylmethionine (AdoMet) most prokaryotic 5-methylcytosine (5mC) DNA methyltransferases increase the frequency of deamination of cytosines targeted for methylation [6] or at 5mCs present in the DNA [7,8].

The source of the DNA undergoing degradation and modifications by sperm cells can be exogenous. In the case of mature sperm cells, exogenous DNA binds to the surface of the cells and becomes internalized. Internalized DNA can be very efficiently cleaved by potent calcium-dependent endonucleases [9]. The 5-mdCMP generated by DNA degradation and reincorporated into replicating fertilized oocytes could have disastrous consequences for embryonic development. This can be prevented by converting 5-mdCMP into dTMP. Here we show that mature sperm cells have a 5-mdCMP deaminase which converts 5-mdCMP into dTMP, which is further cleaved into thymine and an abasic sugar. In addition, we also show that the purified enzyme preparation free of nuclease deaminates 5mC present in double-stranded DNA. The implications of these findings are discussed.

2. Materials and methods

2.1. Preparation of human sperm extracts

Fresh human sperm was processed as outlined by Tash and Means [10]. Sperm cells were lysed into 20 mM HEPES, pH 7.5, 1 mM $MgSO_4$, 1 mM dithiothreitol and 0.4 M KCl. After centrifugation at $30\,000\times g$/15 min at 4°C, the supernatant fraction was collected and frozen in aliquots at −80°C. The chromatin sediment was consecutively re-extracted with 1 M KCl and 2 M KCl in the above buffer. After centrifugation, the supernatant fractions were aliquoted and frozen at −80°C.

2.2. Preparation of labeled DNA substrates

The oligonucleotide duplex A (only the upper stands are shown) 5′-TAGCTGACGATACACATTGATCGGTGATCGTTTCAATATC-CGGCCGTAT-3′ was methylated at CpG with tritiated AdoMet and *Sss*I DNA methyltransferase as outlined by the manufacturer. The oligonucleotide duplex B 5′-TAAACGATTACGTTTACGAATTAT-CGTATATACGATGTGTATCCTCAGCTA-3′ was labeled at the cytosine with deoxy [1′, 2′, 5^3H]cytidine 5′ triphosphate and the Klenow enzyme. Oligonucleotide 5′-TAGCTGAGGATACAC-3′ was used as a primer.

2.3. Preparation of labeled 5-mdCMP (3′ or 5′)

The methylated deoxy nucleotide monophosphates labeled either at the methyl group or at the cytosine were obtained by enzymatic cleavage of the labeled oligonucleotide duplexes. The nucleotide 5′-monophosphates were obtained by a digestion with nuclease P1 whereas nucleotide 3′-monophosphates were obtained by a digestion with micrococcal nuclease. Great care had to be taken when digesting with these two nucleases. Depending on the batches of enzyme, they both effectively cleaved the base from the sugar-phosphate. However, neither the P1 nor the micrococcal nuclease has any trace of 5-mdCMP deaminase activity.

The nucleotide monophosphates were then purified by cellulose thin layer chromatography (TLC) using butyric acid, ammonia and H_2O (66:1:33) as the solvent. Suitable carriers were spotted for UV detection. Spots were cut out, and the labeled deoxy nucleotide monophosphates were eluted with water at 50°C and lyophilized.

2.4. Preparation of labeled 5mC and 5-methyldeoxycytidine

[^{3}H]5mC was obtained by acid hydrolysis of 5-mdCMP. Labeled 5-mdCMP dissolved in 98% formic acid was sealed in a glass capillary

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and hydrolyzed for 90 min at 140°C. The reaction product was lyophilized and the dry sediment dissolved in dimethyl sulfoxide (DMSO). 5mC was purified by TLC on cellulose using butyric acid, ammonia and H_2O (66:1:33) as the solvent. [^{3}H]5-methyldeoxycytidine was obtained by a digestion of 5-methyldeoxycytidine 5′-monophosphate with calf intestine alkaline phosphatase. The reaction product was purified by TLC on silica gel using chloroform and methanol (80:20) as the solvent.

2.5. Analysis of bases, deoxy nucleosides and deoxy nucleotides

Thymine, 5mC, 5-methylcytidine, cytosine, uridine and uracyl were separated by TLC on silica gel using chloroform and methanol (80:20) as the solvent. All nucleotide monophosphates were separated by TLC on cellulose, using butyric acid, ammonia and H_2O (66:1:33) as the solvent.

2.6. Assay of 5-mdCMP deaminase

5 ng of labeled substrate were incubated in 50 μl of 20 mM HEPES, pH 7.5, 1 mM EDTA containing an aliquot of the protein fraction (10 ng–10 μg) to be tested. Following an incubation of 5 h (or overnight) at 37°C, samples were lyophilized and dissolved in 10 μl of either water or DMSO. Aliquots of 1 μl were analyzed for the reaction products using TLC on either silica gel or cellulose plates.

2.7. Test of 5-methylcytidyl-DNA deaminase

For this particular test it was essential that the sperm extract was free of nucleases. This was achieved by chromatography of sperm extracts on a mini Q column. The oligonucleotide duplex A labeled with *Sss*I methyltransferase and [^{3}H]AdoMet was incubated in 50 mM NaCl, 20 mM HEPES, pH 7.5, 1 mM EDTA, 50 μg bovine serum albumin in 40 μl in the presence of 10 ng purified deaminase. After 20 h incubation at 37°C the samples were split into two aliquots. One aliquot was directly lyophilized and dissolved in 10 μl H_2O and spotted onto TLC plates. This sample was for the background determination of non-specific release of thymine and dTMP due to the combined action of residual nucleases and deaminase. The other aliquot was lyophilized, dissolved in 20 μl of 30 mM sodium acetate, pH 5.5, and digested for 1.5 h with 5 U of nuclease P1. Samples were then lyophilized and dissolved in 10 μl H_2O and separated on TLC plates. The counts released by the non-digested sample (residual nuclease activity) were subtracted from the counts present in thymine+dTMP released by the nuclease P1. As a positive control, [^{3}H]5-mdCMP was incubated under the same conditions as above with purified deaminase and the reaction products were analyzed using TLC. Since we could argue that the thymin released during the 1.5 h incubation with the nuclease P1 could represent residual deaminase activity, it was necessary to carry out an additional control. For this purpose, the purified enzyme was incubated overnight in the absence of DNA substrate. After lyophilization the sample was tested for residual deaminase activity. The dry sediment was dissolved in 10 μl of 50 mM sodium acetate buffer, pH 5.5, containing the standard substrate [^{3}H]5-mdCMP and 5 U of nuclease P1. After incubation for 1.5 h at 37°C, aliquots of 1 μl were spotted onto TLC plates and tested for the conversion of 5-mdCMP into dTMP and the release of thymine. In the negative control the total conversion and release of dTMP+thymine did not exceed 3–5% of the input counts of the labeled substrate.

2.8. Purification of the 5-mdCMP deaminase

The crude sperm extracts were dialyzed against 20 mM HEPES, pH 7.5, and 50 mM NaCl. 300 μg of dialyzed proteins were loaded onto a 250 μl mini Q column (Smart system, Pharmacia) using step elution with increasing salt (NaCl) concentrations. The deaminase activity eluted as a main peak at 0.15 M NaCl. The purified fractions were unstable at −80°C but stable for weeks when kept in liquid nitrogen. The active fractions were further analyzed on 10% SDS–polyacrylamide gels with silver staining. Preparative SDS–PAGE (10%) gel electrophoresis was carried out as previously described [11].

2.9. Chemicals and enzymes

Klenow enzyme and nuclease P1 were purchased from Roche. *Sss*I methyltransferase came from Biolabs and micrococcal nuclease from Sigma. [^{3}H]AdoMet (73 Ci/mmol) and deoxy [1′, 2′, 5^{3}H]cytidine 5-triphosphate (64 Ci/mmol) were purchased from Amersham. Silica and cellulose plates were from Merck. 5-methyl 2′-deoxycytidine, 5-

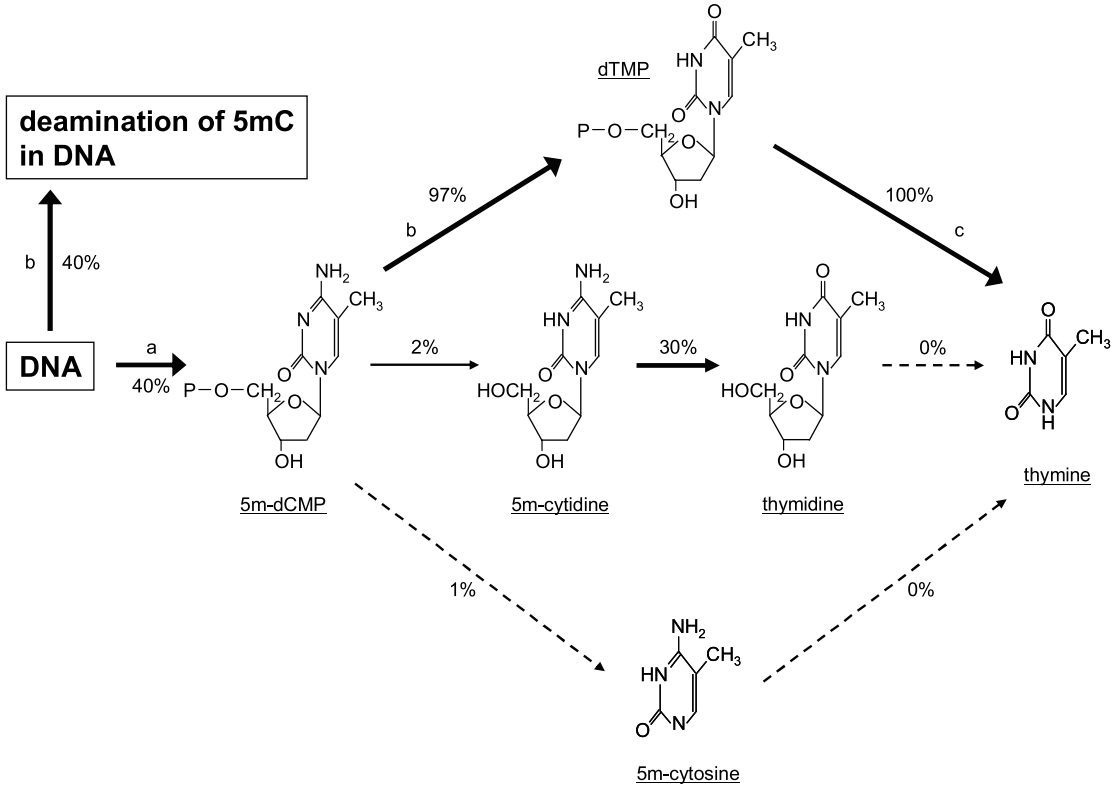

Fig. 1. Pathway of deamination (sperm extracts) of 5mC in intact DNA and in 5-mdCMP generated by nuclease degradation of labeled DNA. a: The nuclease degradation step; b: the deamination reaction; c: the cleavage of the deaminated base from the sugar-phosphate. The percentage conversion for each step was determined in vitro with either crude sperm extracts or with purified fractions of deaminases free of nucleases.

methyl 2′-deoxycytidine monophosphate, thymine, thymidine, thymidine 5-monophosphate, uridine, and uracyl were purchased from Sigma. Cytidine and cytosine were from Fluka. 5mC and deoxycytidine 5′-monophosphate were from Serva.

3. Results

3.1. [^{3}H]5mC or [^{3}H]C labeled DNA incubated with sperm extracts is degraded to give [^{3}H]thymine and [^{3}H]uracyl respectively

It has been shown that mature sperm cells contain high levels of endonucleases [6]. Fig. 1 shows the degradation and reaction pathways that we have established for methylated DNA incubated with total or fractionated human sperm cell extracts. In vitro the labeled DNA is either degraded to mononucleotide phosphates, which are deaminated, or the deamination can also occur in vitro with intact labeled DNA. As shown in Fig. 2, up to 40% of 5mC and C present in the labeled double-stranded oligonucleotides incubated with non-purified sperm extracts was recovered as free thymine (solid line) and uracyl (dotted line) respectively. Fig. 2 also shows that there are no apparent free intermediary products such as 5mC (a), 5-methylcytidine (b) or dTMP (c) liberated from the degraded DNA. On denaturating gels, the DNA incubated with the non-fractionated sperm extracts is extensively degraded and the nucleases do not show any base specificity (data not shown). What is/are the substrate(s) of deamination: the degradation products of the DNA or the intact DNA? In order to answer this question, oligonucleotides labeled at 5mC were incubated with non-fractionated sperm extracts in the presence of a vast excess of non-labeled 5-mdCMP or dTMP. Fig. 3 (panel B) clearly shows that in the presence of 5-mdCMP (or dTMP, result not shown) there is an accumulation of [^{3}H]5-mdCMP which may serve as a substrate for the deamination. The positive control in panel A shows that incubation in the absence of non-labeled 5-mdCMP gives no accumulation of 5-mdCMP. Fig. 4 shows that 5-mdCMP is indeed the substrate of deamination resulting from DNA degradation, and 5-mdCMP is a better deamination substrate than dCMP.

Moreover, there is no difference in the extent of deamination between the 5′ or the 3′ monophosphates (Fig. 4, asterisks). When labeled 5mC was incubated with sperm extracts, no deamination was observed (Fig. 5A). Moreover, labeled 5-methylcytidine is a poor substrate of deamination that cannot be converted to thymine (Figs. 5B and 1).

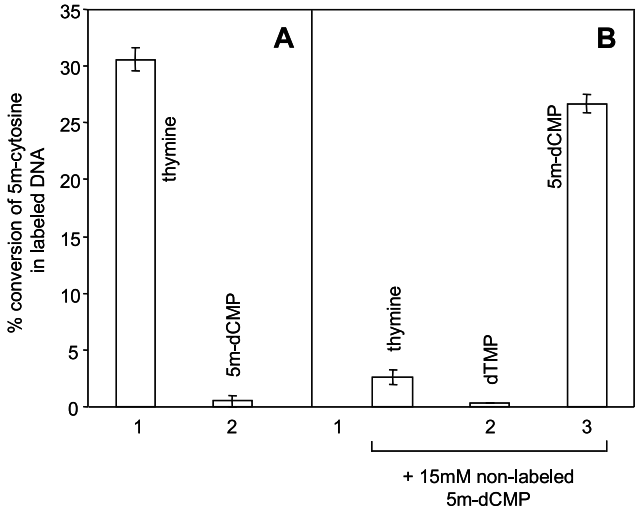

Fig. 3. The deamination substrate resulting from nuclease degradation of DNA is 5-mdCMP. Oligonucleotide duplex A labeled at 5mC was incubated for 15 h (300 000 cpm) in the presence of 10 μg sperm extracts (panel A). In panel B the same reaction was carried out but with the addition of 15 mM non-labeled 5-mdCMP. The results are expressed as percentage conversion of the 5mC in the labeled oligonucleotide duplex A.

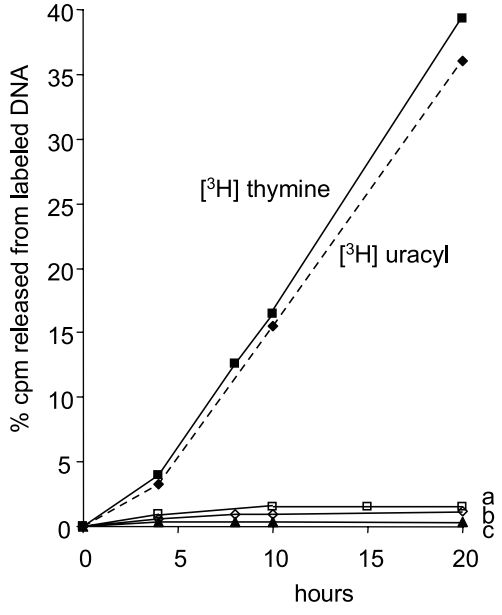

Fig. 2. Kinetics of degradation of labeled oligonucleotides with sperm extracts. [^{3}H]Thymine was released from the degraded nucleotide duplex A labeled at 5mC. [^{3}H]Uracyl was released from oligonucleotide B labeled with [^{3}H]dCTP. For each time point 200 000 cpm substrates were incubated with 10 μg sperm extracts. a, b and c are the released 5mC, 5-methylcytidine and dTMP respectively.

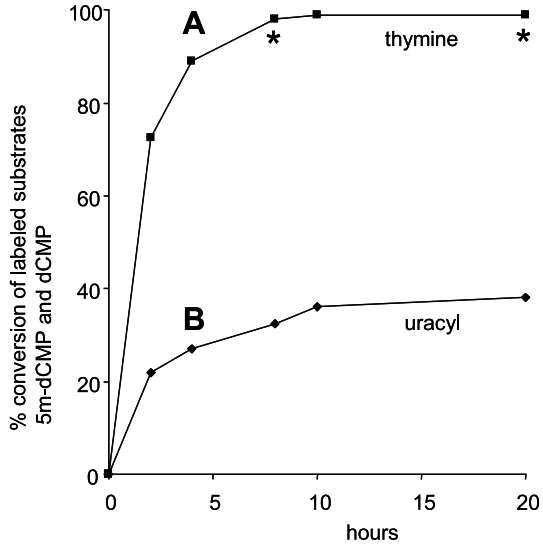

Fig. 4. Curve A represents the kinetics of the deaminase reaction with labeled 5-methyl deoxycytidyl-5′-monophosphate (150 000 cpm per time point) as substrate. The asterisk represents the release of thymine from 5-methyl deoxycytidyl 3′-monophosphate (150 000 cpm per time point). In curve B, dCMP (150 000 cpm per timepoint) is the substrate. The reaction was carried out as outlined in Section 2. Each time point is the average of three measurements.

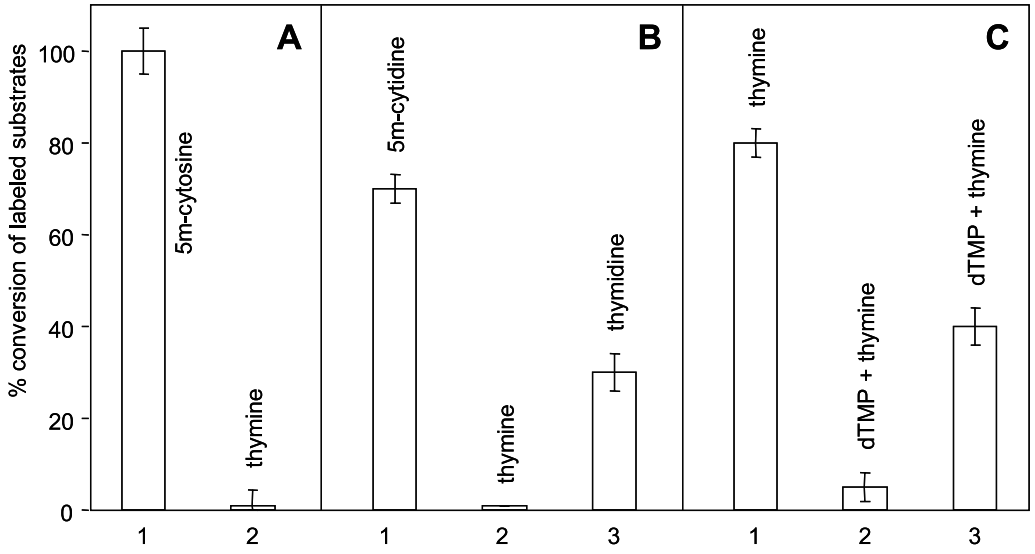

Fig. 5. Substrate specificity of the deaminase from sperm extracts. Panel A, the substrate 5mC (200 000 cpm) was incubated for 15 h at 37°C with 10 μg of sperm extract. Panel B, 5-methylcytidine (150 000 cpm) was incubated for 15 h with 10 μg sperm extract. Panel C is the test of 5-methylcytidyl-DNA deaminase. The first bar is the positive control of labeled 5-mdCMP (150 000 cpm) incubated with 10 ng purified deaminase free of nuclease (fraction 0.15 M NaCl from mini Q column) for 20 h at 37°C. The second and third bars show the incubations with the oligonucleotide A labeled at 5mC (150 000 cpm) incubated with 10 ng purified enzyme. The second bar shows the control not treated with nuclease P1 and represents the residual nuclease in the purified enzyme. In the third bar the samples were treated with nuclease P1 after incubation in order to release all nucleotide monophosphates and the specific release of dTMP+thymine was analyzed by TLC. Each assay was run in triplicate.

3.2. A nuclease-free fraction of 5-mdCMP deaminase deaminates in vitro methylated DNA

In sperm extracts, the very high activities of nucleases preclude any study of the possible deamination of 5mC present in double-stranded DNA. It was therefore necessary to purify the enzyme free of nucleases. This was achieved by chromatography of the sperm extracts on a mini Q column (Smart system, Pharmacia). Fig. 6 shows an elution profile (step elution with NaCl) of 300 μg sperm extract loaded on a 250 μl mini Q column. 5-mdCMP deaminase and dCMP deaminase activities co-eluted with 150 mM NaCl (Fig. 6). This fraction of proteins was essentially free of nucleases and the majority of the nucleases were found in the flow-through fraction (result not shown). Another broad shoulder of dCMP deaminase activity eluted between 200 and 400 mM NaCl. The fraction eluted with 150 mM NaCl was tested for the presence of 5-methylcytidyl-DNA deaminase activity. The first bar in Fig. 5C is a positive control and shows the deamination of the standard substrate 5-mdCMP incubated with 10 ng of purified deaminase. The second bar (Fig. 5C) is the control where methylated double-stranded DNA was incubated with the purified deaminase under the same conditions as in the first bar. It shows essentially no release of deaminated reaction product from the DNA, thus confirming the absence of nucleases in this fraction. Should the reaction mixture shown in the second bar be treated with nuclease P1, then a substantial release of deamination products (dTMP and thymine) can be observed (see the third bar of Fig. 5C). The liberation of [^{3}H]thymine after incubation of the reaction mixture with nuclease P1 is due to the ability of the nuclease P1 preparation to cleave the glycosydic bond between thymine and the sugar-phosphate (see Section 2). However, under our test conditions the nuclease P1 showed no trace of deaminase activity, thus ruling out a possible artifact of the reaction.

3.3. Characterization of 5-mdCMP deaminase

Further purification of the fraction eluted from the mini Q column was achieved by preparative (10% or 11%) SDS–PAGE. Fig. 7 shows that the main activities of 5-mdCMP and dCMP deaminases were recovered in the lower part of the gel corresponding to an apparent molecular weight (MW) of about 25 kDa. Another peak of activity was also recovered at the gel position of about 80 kDa. This peak may represent an incomplete dissociation of the protein complex containing the deaminase. When tested with the 5-mdCMP substrate, the deaminase had a K_m of 1.4×10^{-7} M with a V_{max} of 7×10^{-11} mol/h/μg protein (Fig. 8). For its full activity, the 5-mdCMP deaminase did not require any divalent cations and is not influenced by the addition of deoxynucleotides triphosphate to the incubation mixtures (data not shown).

4. Discussion

Two different kinds of 5mC deaminase activity are present in the human mature sperm cells. One deaminates 5-mdCMP generated by the nuclease degradation of DNA and the other deaminates 5-methylcytidyl residues present in intact double-stranded DNA. The two activities are carried out by the same purified enzyme preparation. As mentioned above [9], mature sperm cells have very high nuclease levels. These nucleases are thought to be important for eliminating any exogenous DNA that may contaminate the sperm cells [9]. Any reincorporation of methylated cytidyl phosphate into the DNA of the rapidly dividing early embryonic cells could possibly silence genes at random and jeopardize the epigenetic imprint. The presence of the 5-mdCMP deaminase could possibly prevent any faulty methylation by deaminating 5-mdCMP into dTMP.

The purpose of a deaminase that can deaminate 5mC present in intact double-stranded DNA is less evident. When

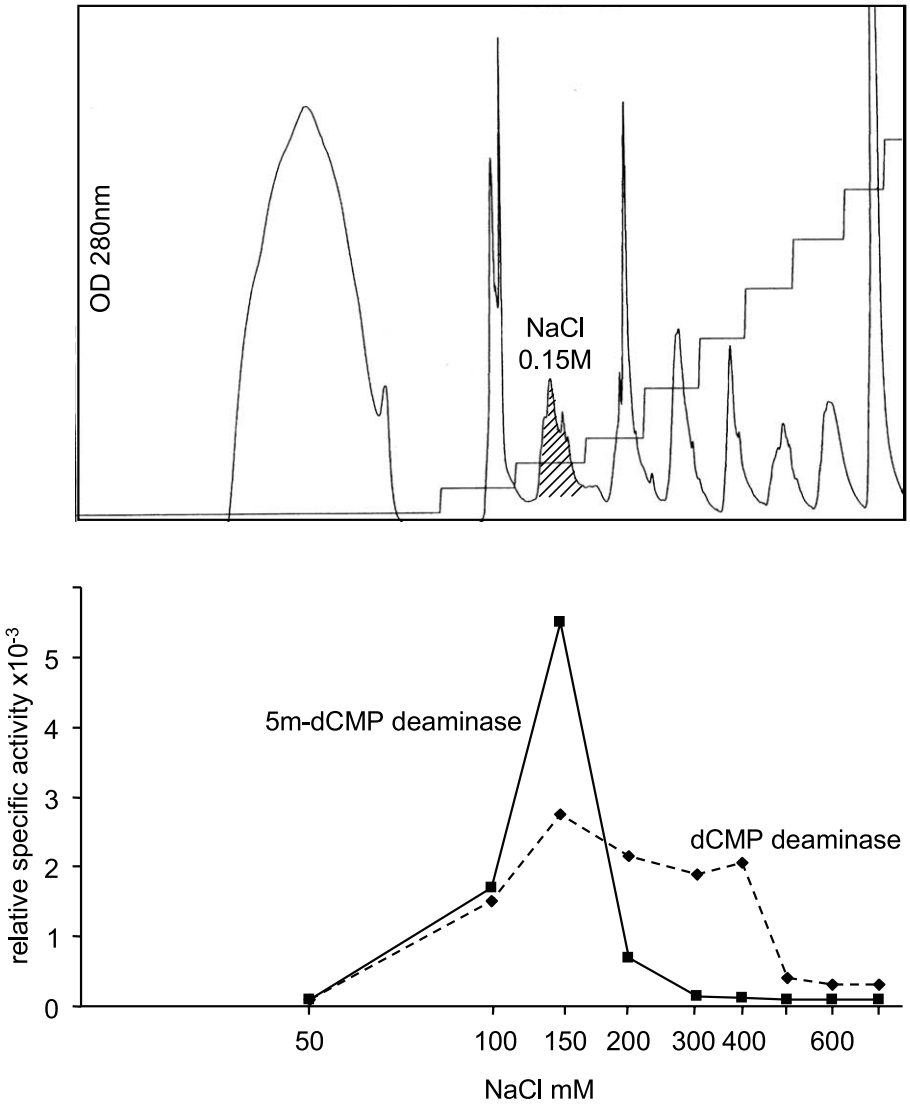

Fig. 6. Chromatography of 300 μg sperm extract (fraction 0.4 M KCl) on a 250 μl mini Q column (Smart system, Pharmacia). The upper panel is the elution profile with an NaCl step gradient. The lower panel represents the activity profile of the 5-mdCMP deaminase (solid line) and the dCMP deaminase (dotted line) eluted with increasing concentrations of NaCl.

operational, the reaction could create mutation hot spots if not immediately repaired; in addition it could generate double-strand breaks in the DNA. The question is: could this deaminating activity be involved in paternal genome-wide demethylation? This reaction would only be possible in the presence of a very efficient DNA repair mechanism. In recent years it has been conclusively shown that shortly after fertilization there is a paternal genome-wide demethylation occurs in the absence of replication [12,13]. So far, no mechanism that could explain this phenomenon has been described. However, in a different context we have previously shown that 5mC-DNA glycosylase (alias G/T mismatch DNA glycosylase) is involved in the demethylation of a limited and defined number of mCpGs in two different systems [14,15]. As determined by our very sensitive assay in the sperm extracts, there is no trace of 5mC-DNA glycosylase (activity measurements and detection by Western blots, Jost, unpublished results). Furthermore, all attempts to detect the demethylase described by Bhattacharya et al. [16] in sperm cells and oocytes were negative. The existence of a demethylase cleaving the carbon–carbon bond of the methyl group of methylcytosine is very attractive but still controversial since the results have not yet been reproduced by other groups [17]. In addition, no modification of the 5mC, such as the formation of 5-hydroxymethyl C, could be detected when DNA was incubated with sperm extracts or combined with oocyte extracts (Jost, unpublished results). As mentioned above, there is a remote possibility that the deamination of 5mC in DNA, combined with a very efficient DNA repair system provided by the oocytes for example [18,19], may be fundamental to paternal genome-wide demethylation. However, one of the big dangers of such a mechanism is the generation of mutations and double-strand breaks. In this context, it is interesting to note that the sperm extracts have high levels of the Ku protein (MW 70 and 80 kDa) which should prevent double-strand breaks ([20]; Jost, unpublished results). In a very different context, it has recently been shown that the deamination of cytosines in DNA plays a very important role in the generation of the variable region of genes coding for antibodies [21,22]. The somatic hypermutation in this case is represented by a high frequency of point

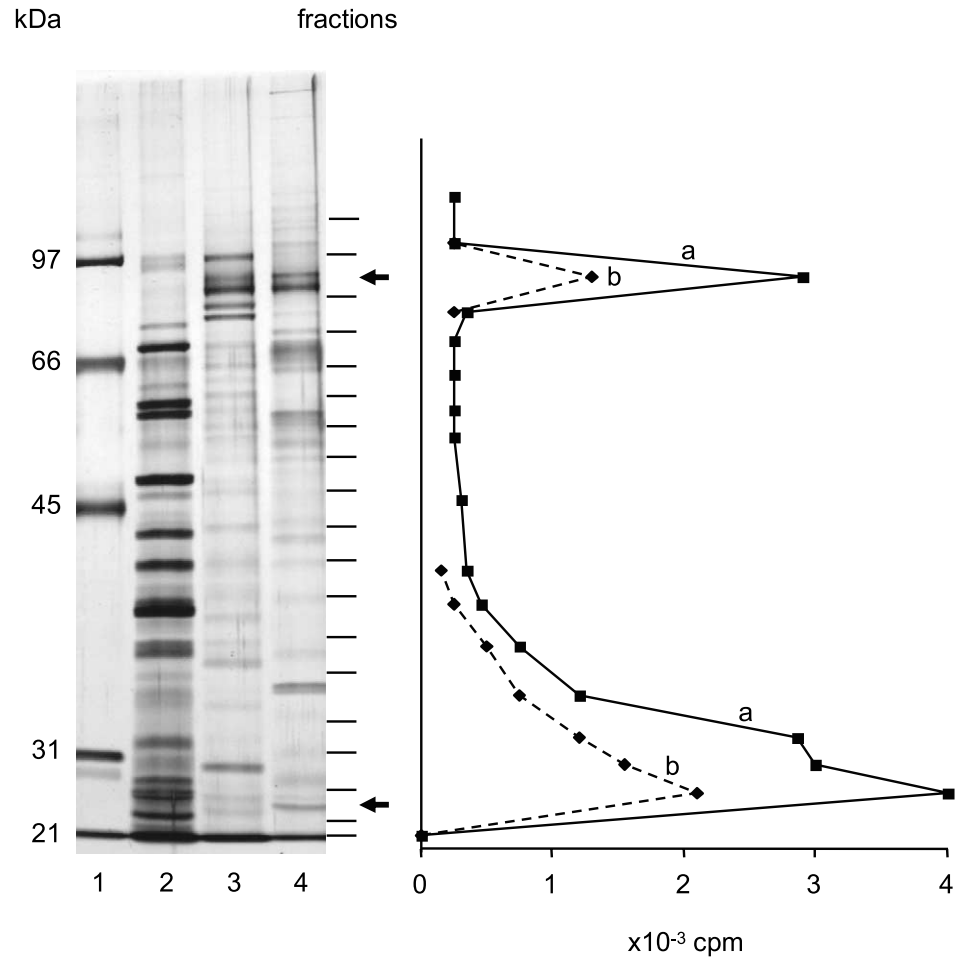

Fig. 7. Preparative 10% SDS–PAGE gel. About 0.5 μg of the purified fraction (0.15 M NaCl, mini Q) was separated by SDS–PAGE (in the cold room). The gel was stained with $CuCl_2$, destained, sliced and protein eluted as previously described [11]. The panel on the left represents a silver stain of eluted fractions from mini Q column. Lane 1 is the size standard. Lane 2 shows the flow-through of the mini Q column. Lane 3 shows the fraction eluted with 0.1 M NaCl, and the active fraction 0.15 M NaCl is in lane 4. The right hand panel represents the activity profiles of (a) mdCMP deaminase and (b) dCMP deaminase eluted from lane 4 of the left hand panel.

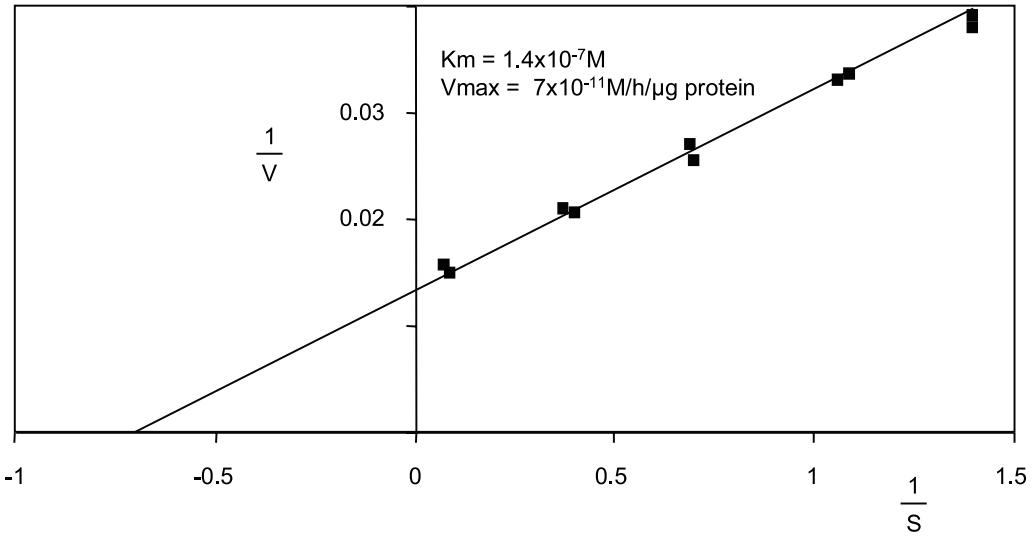

Fig. 8. Determination of the K_m and V_{max} of 5-mdCMP deaminase present in human sperm crude extracts. The substrate is labeled 5-methyl deoxycytidyl 5′-monophosphate and the assay was carried out as outlined in Section 2. Results are expressed as a Lineweaver–Burk plot.

mutations occurring only during the outburst stage of B cell differentiation. For this process, an activation-induced cytidine deaminase is required [21,22]. A cytidine deaminase that may be responsible for this reaction has been isolated and cloned from B cells [23,24]. As for the deaminase presently described here the deaminase from B cells also has a MW of about 24 kDa.

Whether the 5-methylcytidyl-DNA deaminase activity in this study plays a role in paternal genome-wide demethylation remains to be demonstrated. In the interim, we should be open to any other possibly unsuspected reactions that may explain the enigmatic reaction of paternal genome-wide demethylation.

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