KINETIC ANALYSIS OF O^6-ETHYLGUANINE DISAPPEARANCE FROM DNA CATALYZED BY THE CHROMATIN FACTOR OF RAT LIVER

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1. Introduction

Rat liver chromatin contains a factor which induces the disappearance of O^6-ethylguanine from DNA alkylated with ethylnitrosourea [1,2]. The transformation product has not yet been isolated nor, of course, identified. We noticed that, in contrast to the situation in *Escherichia coli* [3,4], this factor is present constitutively in chromatin and that it seems to be an enzyme rather than a stoichiometric reagent: it is inactive at 0°C and, when there is an excess of substrate, it is still working after 120 min at 37°C.

Here, we give additional details on the chromatin repair factor showing that it behaves as an enzyme which is competitively inhibited by the reaction product.

2. Materials and methods

2.1. *Chromatin proteins*

Rat liver nuclei and chromatin are isolated according to [5]; the proteins are prepared using heparin–Sepharose as in [2], except that the heparin–Sepharose–DNA–protein complex is eluted with 0.3 M KCl, 10 mM K-phosphate, 10 mM Tris–HCl (pH 8.0). This extract is dialyzed against buffer A (20 mM Tris–HCl; 1 mM EDTA, 0.2 mM dithiothreitol, pH 8.0, 10% glycerol).

2.2. *Substrate*

Calf thymus DNA (Sigma) is alkylated with [^{3}H]-ethylnitrosourea (3 Ci/mmol; IRE Belgium). The ethylated DNA, partially depurinated according to [4], contains 7.8 O^6-ethylguanine residues/10^6 guanines. It is kept in buffer B (10 mM Tris–HCl, 1 mM EDTA, pH 8.0).

2.3. *Purine analysis*

To the ethylated DNA in 200 μl incubation medium are added 50 μl 10 mM HCl containing 50 μg unlabelled O^6-ethylguanine and 26 μl 1 M HCl; the mixture is warmed at 70°C for 50 min. After addition of 50 μl 2.5 M ammonium formate and 120 μl ethanol:acetonitrile (65:35, v/v), the precipitate is discarded by centrifugation and the supernatant (400 μl) is chromatographed on a 25 × 0.9 cm column of Partisil 10 ODS-2 (Whatman) using an Altex 332 HPLC system. The eluent is a mixture of 10% acetonitrile and 90% of a solution constituted of 4 vol. 25 mM ammonium formate adjusted at pH 4.00 with formic acid and 1 vol. ethanol. The elution is performed at a rate of 2.5 ml/min and the pressure is 1000–1500 lb/in^2. The analysis takes 15 min and the retention time of O^6-ethylguanine is 10.8 min; a 2.5 min fraction is collected after 10 min. The A_{280} enables one to calculate the yield of O^6-ethylguanine recovery (80–90%). The total radioactivity of the fraction is corrected for the yield; taking account of the specific radioactivity of the [^{3}H]ethylnitrosourea used to prepare the substrate, one calculates the amount of O^6-ethylguanine (fmol) present in the ethylated DNA before and after the action of the repair factor. The standard error of the analysis for the substrate DNA is 1–2%.

3. Results

Aliquots (100 μl) of chromatin proteins (58 μg) in buffer A are mixed with 100 μl buffer B containing 67, 134, 347 or 660 fmol O^6-ethylguanine in DNA; the incubation at 37°C is stopped at different times up to 120 min before looking for the remaining O^6-ethylguanine. Fig.1 shows a complete (67 and 134

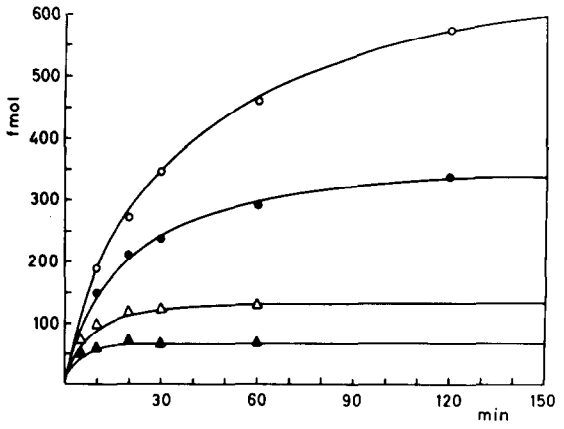

Fig.1. Kinetics of O^6-ethylguanine disappearance from DNA. The same amount of chromatin proteins (58 μg) is incubated at 37°C with different amounts of ethylated DNA ((▲) 67, (△) 134, (●) 347; (○) 660 fmol O^6-ethylguanine) in 200 μl buffer for various times up to 120 min. The ordinates (fmol) indicate the amount of O^6-ethylguanine that has disappeared from DNA. Symbols are experimental results; the theoretical curves are drawn using V_{max} = 37 fmol/min, K_m/K_i = 4.7, K_i = 5 pM and the equation given by [7].

fmol substrate) or nearly complete (347 and 660 fmol) disappearance of O^6-ethylguanine from DNA when the incubation time is sufficiently long.

If the slopes of straight lines joining the origin with the first experimental point of each progress curve in fig.1 (or in other experiments, not shown) are taken as initial velocities which are subsequently plotted against substrate concentration either directly or in Lineweaver-Burk coordinates, an apparent Michaelis constant (K_m) is obtained of <50 pM; this corresponds to <10 fmol in the 200 μl incubation medium. If one looks now at the progress curve corresponding to 660 fmol, one sees that the reaction velocity slows down continuously from the beginning although the initial substrate concentration is many times this app. K_m.

One possible explanation is that the enzyme is progressively inactivated during the incubation. To test this hypothesis, the chromatin proteins (43 μg) in 100 μl buffer A plus 50 μl buffer B is preincubated at 37°C for various times up to 120 min; 50 μl buffer B containing the substrate is then added and incubated for 10 more min at the same temperature before analyzing the DNA. The logarithm of enzyme activity (fmol O^6-ethylguanine removed from DNA, which is proportional to the amount of enzyme [2]) plotted as a function of time gives a straight line (not shown), the slope of which indicates a half-life of 55 min for the free enzyme at 37°C.

But the enzyme is not free when the substrate concentration is many times the K_m. The stability of the enzyme in the enzyme–substrate complex is thus explored by the method in [6]: if the enzyme in the complex is stable, product formation is the same function of time multiplied by enzyme concentration whatever this latter concentration may be. In fig.2, three different amounts of chromatin proteins (10, 20 and 40 μg of another preparation) are used in otherwise identical incubation media. Disappearance of O^6-ethylguanine is taken as equivalent to product formation. The 3 curves are different indicating that the enzyme is labile. The lateral displacement of the curve for a given amount of product formation with decreasing amount of enzyme enables to calculate a half-life of 85 min for the enzyme in the enzyme–substrate complex at 37°C.

Enzyme inactivation is however not sufficient to account for the slowing down of the reaction with time. Indeed, if one were to make the supposition that it is the only cause, a half-life of 15 min would give the best fit for the curve of fig.2 corresponding to 40 μg protein; applying this half-life to 20 and 10 μg protein would yield theoretical curves which are very different from the experimental ones (fig.2).

The results are qualitatively but not quantitatively reproducible; the half-lives of free enzyme and enzyme in the enzyme–substrate complex depend on the chromatin preparation. In spite of the presence of an inhibitor of serine-proteases (phenylmethylsulfonyl fluoride) during the preparation of the chromatin extract, there is probably some residual proteolytic activity the importance of which differs from one preparation to the next. However enzyme degradation is always a minor cause of the decreasing reaction rate so that it will be neglected in further analysis of the reaction kinetics.

The slowing down as the reaction proceeds may be due to product inhibition. If the inhibition is competitive, plotting $(S_o - S)/t$ against $(1/t)\ln(S_o/S)$ gives a straight line (S_o = initial substrate concentration; S = substrate concentration at time t), the slope of which is equal to $K_m\ (K_i + S_o)/(K_m - K_i)$, where K_m and K_i are respectively the Michaelis constant and the inhibition constant [7]. Fig.3A, which presents data from fig.1 for the 3 higher O^6-ethylguanine concentrations, shows that it is indeed the case; the slopes are positive which indicates that $K_m > K_i$. When these

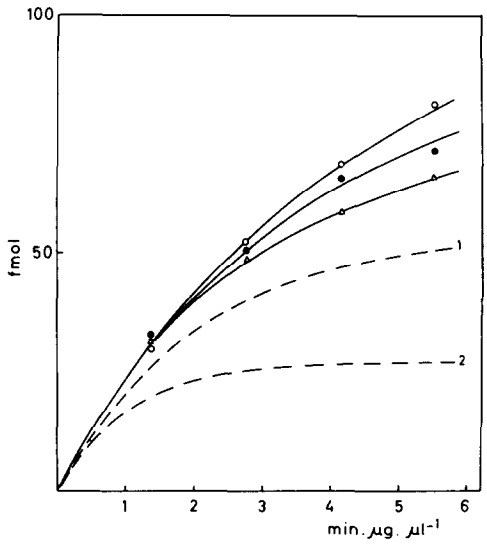

Fig.2. Instability of enzyme in the enzyme–substrate complex. The same amount of ethylated DNA (347 fmol O^6-ethylguanine) is incubated at 37°C with 10 μg (△), 20 μg (●), or 40 μg (○) chromatin proteins from the same preparation in 200 μl buffer. The ordinates (fmol) show the amount of O^6-ethylguanine that has disappeared from DNA; the abscissae give time multiplied by protein concentration (min . μg . μl^{-1}). The continuous lines are best curves drawn through the experimental results. Making the hypothesis that the decreasing reaction rate when 40 μg protein are present is due only to enzyme inactivation, one calculates a half-life of 15 min; applying this half-life to the lower protein concentrations leads to the discontinuous theoretical curves ((1) 20 μg; (2) 10 μg protein)).

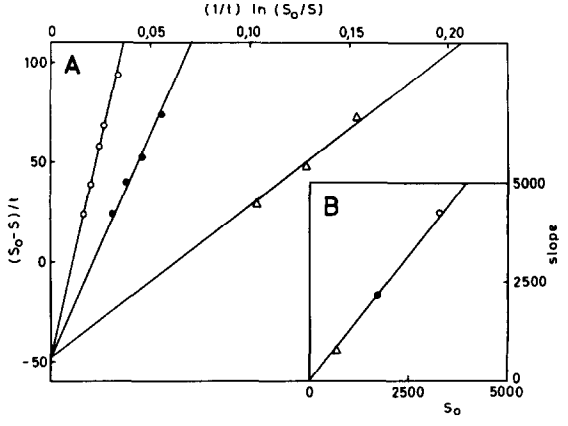

Fig.3. Estimation of V_{max} and K_m/K_i ratio. (A) Results from fig.1 ((△) 134, (●) 347, and (○) 660 fmol O^6-ethylguanine). S_0 and S = O^6-ethylguanine concentrations (pM) at the beginning of the incubation and at time t (min), respectively. (B) Slopes (pM) of straight lines from (A) versus S_0 (pM).

slopes are plotted against S_0, another straight line is obtained (fig.3B) having a slope of $K_m/(K_m - K_i)$ = 1.27, wherefrom one calculates K_m/K_i = 4.7. Since the intercepts of the straight lines in fig.3A with the ordinate axis yield $V_{max}/(1 - K_m/K_i)$, one calculates V_{max} = 37 fmol O^6-ethylguanine removed from DNA per minute for the 58 μg chromatin protein.

Theoretically K_i can be obtained from fig.3B, but it is unreliable because the error is too high. Another approach is to put the known values of V_{max} and K_m/K_i in the equation given by [7] and to fit the experimental progress curves with a computer varying the K_i value. This fitting (fig.1) is improved by decreasing K_i to 5 pM; it stays at the same level for lower values. Thus: $K_i \leqslant 5$ pM, and $K_m \leqslant 25$ pM.

Although the results are always qualitatively the same, the ratio K_m/K_i varies from one experiment to another; we found values scattered between 1 and 6.

4. Discussion

Looking at these data, nobody would have thought that the chromatin protein responsible for the modification of O^6-ethylguanine in DNA might not be an enzyme. There is now the opionion [3] that the *Escherichia coli* factor involved in the removal of O^6-methylguanine from DNA is a stoichiometric reagent, and also the unresolved question of the nature of the reaction product, so that, in our calculations, substrate disappearance is equated to product formation. Can we certify that the chromatin factor is not a stoichiometric reagent?

If the chromatin factor were a stoichiometric reagent, the results presented in fig.1 would mean that, even when the largest amount of substrate was used (660 fmol), the number of repair factor molecules in the incubation medium were in excess of the O^6-ethylguanine residues in DNA since the reaction went nearly to completion; so, when we consider cases with much smaller quantities of substrate, the amount of factor being the same would be in a large excess. In this latter situation, if we imagine that a successful meeting between factor and O^6-ethylguanine immediately leads to the product, the reaction kinetics ought to be first order.

All the O^6-ethylguanine residues in DNA might also immediately form complexes with the repair factor and a subsequent modification of the complex lead to the destruction of O^6-ethylguanine and the simulta-

neous inactivation of the factor. Such a reaction would also be first order.

But plotting the logarithm of the remaining O^6-ethylguanine as a function of time does not give a straight line whatever the substrate initial concentration (not shown); moreover the initial slope decreases when the amount of substrate increases.

One could devise more complicated scenarios and perhaps find one which would fit the experimental results so that, at present, the hypothesis that the chromatin repair factor might be a stoichiometric reagent cannot be ruled out. Final proof will probably have to await complete purification of the repair factor; we shall then verify if one molecule is able or not to lead to the disappearance of more than one residue of O^6-ethylguanine.

It is however much more likely that the chromatin factor that destroys O^6-ethylguanine in DNA is an enzyme which is competitively inhibited by the reaction product. The app. K_m/K_i ratio varies from 1–6 depending on the experiment. The variation is probably due to reactions not taken into account in the model; the reaction product might, for instance, be further metabolized by another protein of the chromatin extract and the relative speeds of the two successive transformations might be different from one chromatin preparation to another. It is obvious that the analysis of the kinetics of O^6-ethylguanine disappearance from DNA ought to be studied again with a purified enzyme.

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