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Temperature dependence of
Strongylocentrotus Purpuratus DNA damage
photoreversal enzymes

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for
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Temperature Dependence of *Strongylocentrotus Purpuratus*
DNA damage photoreversal enzymes

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Abstract

Thermodynamic measurements of the *Strongylocentrotus purpuratus* DNA damage photoreversal enzyme were attempted *in vitro*. Using an Arrhenius plot, the rate of DNA repair at 4, 13, 19, and 25 °C should give the energy of activation for the photoreversal enzyme. This measurement technique yielded a relatively precise value for the activation energy of $-21.6 \pm 0.5$ kJ/mol·K. However, the fact that this has a negative value suggests that this may be, in fact, the activation energy for a tumor suppressor gene. That is, instead of examining the rate of repair, this experiment examines the rate of embryo termination.

Introduction

*S. purpuratus* development, like the development of most organisms, is highly sensitive to UV irradiation. Serious irradiation will normally cause sufficient chromosomal damage to prevent gastrulation. Because UV light is common in aquatic environments, and the consequences of serious DNA damage are so dramatic, many organisms have developed mechanisms for preventing and repairing UV damage. In sea urchins, one major repair system is through the photoreversal enzyme (Akimoto *et. al.*, 1987). While the kinetics and activity periods of this enzyme have been studied generally, little is known about the protein’s thermodynamic properties (Amemiya *et. al.*, 1986; Ejima *et. al.*, 1982).

This paper explores the thermodynamic variability of the photoreversal enzyme’s function for fertilized one-cell embryos of the purple sea urchin (*Strongylocentrotus purpuratus*). To calculate an activation energy value ($\Delta G$), the Arrhenius equation is applied to enzyme rates at various temperatures:

$$ r = e^{-\frac{\Delta G}{R \cdot T}} $$

$$ \ln r = -\frac{\Delta G}{R} \cdot \frac{1}{T} $$

where $r$ is the rate of mutation repair, $\Delta G$ is activation energy for DNA repair, $R$ is the universal gas constant, and $T$ is the temperature in Kelvin.
The translation of this equation to living systems involves some subtlety, however. Because it is difficult to visualize individual DNA repair events, on the scale that this enzyme functions, some assumptions about DNA damage must be made. In this experiment, the number of cells reaching gastrula stage is used to measure the relative amount of damage. The problem is that exposure to UV radiation usually results in many specific mutations, only a few of which are fatal. In a sample where less than half of the cells are fatally mutated, we can assume that most of those that die, succumb to a single fatal mutation. Mathematically, if \( p \) is the probability of one cell sustaining a UV mutation then the number of fatally mutated cells is:

\[
\sum_{n=1}^{\infty} p^n
\]

Thus, if half of the cells in a population are killed, exactly two-thirds of the cells would have had only one mutation (i.e. \( \sum_{n=1}^{\infty} \left(\frac{1}{3}\right)^n = \frac{1}{2} + \frac{1}{6} + \frac{1}{18} + \cdots = \frac{2}{3} \)). By using a short UV exposure time to keep the number of fatal mutants to less than one-half, the assumption that only single fatal mutations are observed becomes valid. Exposure times of around 30 seconds should produce a noticeable number of fatal mutants without killing more than half of the embryos (Akimoto et al., 1987).

To observe these mutations, it is important to have some understanding of the stages of embryonic development. Egg fertilization, for example, results in changes that are easily observable. Approximately sixty seconds after sperm bind to receptors on the egg membrane, the fertilization envelope becomes visible surrounding the egg. This envelope prevents polyspermy, and is formed as Ca\(^{++}\) ions trigger egg cortical granules to fuse with the plasma membrane, creating a layer between the egg plasma membrane and the vitelline layer. The observation of this layer before irradiation is critical in establishing the condition of the embryos prior to the irradiation.

Following formation of the fertilization envelope, cells begin to cleave every one to two hours for approximately 18 hours. During this stage, it is actually difficult to observe any mutations caused by irradiation because, at this point, protein synthesis is not critical to development. After about 18 hours, embryos undergo blastulation. As blastula, the cells develop cilia and begin to spin in place. This stage is followed by gastrulation, where the activation of protein synthesis causes
mutations in the DNA become apparent. An invagination of the cells on the vegetal pole of the gastrula causes the cells to move from the action of the cilia on the animal pole. In cells with critically mutated genomes, this stage is never reached. Thus, it is after 24-48 hours following fertilization that the effects of the DNA repair enzymes can be observed.

Given that the ratio of embryos surviving to gastrulation can be directly related to the success of the DNA repair enzyme, this experiment examined the gastrulation rates as a function of temperature. To control for the effects of temperature on early embryo development two sets of embryos were kept at differing temperatures for one hour following exposure to UV radiation. One of these embryos was exposed to visible light while the other was wrapped in foil. After one hour, the effects of the photoreversal enzyme are negligible, so the embryos were allowed to develop in light at 13°C until gastrulation (Akimoto et. al., 1987). Thus, the only difference between the baseline and experimental rates of gastrulation was the presence of light for activating the photoreversal enzyme. The rate constant is just the difference in gastrulation (or the number of critical mutants fixed per cell) divided by the time the protein took to act. It should be noted that while this time interval is somewhat arbitrarily set at 30 minutes, it is only the relative value of the rate that affects the final slope. By plotting the natural log of the repair rate against the inverse of the temperature, $-\Delta G/R$ for this enzyme is simply the slope of this line.

**Materials and Methods**

Unfertilized eggs were suspended in a natural sea water solution. The eggs were placed on a petri dish and, using a toothpick, sperm was added to the solution. Eggs were then allowed to fertilize for 60 seconds before being centrifuged. The cells were then suspended in a fresh solution of natural sea water. By removing unsuccessful sperm, proteolytic enzymes from these sperm were prevented from degrading the developing eggs. These cells were then checked for fertilization envelopes before proceeding.

Cells were then divided into nine petri dishes. Eight of the dishes were exposed, uncovered, to UV light for 30 seconds at 25°C using a standard tissue culture
UV lamp (Shiroya et al., 1984). Because all dishes were exposed in parallel, the exposures all took place at the same time (about eight minutes) after fertilization.

Four of the eight exposed dishes were wrapped in foil. One pair of wrapped and unwrapped dishes was placed in lighted, temperature-controlled rooms of 4°, 13°, and 19°C. The remaining pair was kept at room temperature along with the unexposed control. The positive control was used to show how development would proceed nominally.

After one hour of ambient light exposure, all dishes were unwrapped. At this point, it is too late for photoreversal to have a significant effect (Akimoto et al., 1987). All dishes were placed in the 13°C cold room for 48 hours.

After 48 hours, the ratio of embryos in gastrulation was observed under a dark field microscope. This difference in the ratio between the exposed and foil-protected samples was then divided by the recovery time to give a recovery rate. The natural log of this rate was graphed against the inverse of temperature to give an Arrhenius plot. ΔG could then be calculated from the slope of this line. Also, z-statistical significance tests were performed on the exposed and protected ratios at constant temperatures.

**Results**

Embryo plates showed marked differences upon examination. The 25°C samples, including the unirradiated control, contained mostly dead, disfigured cell clusters. Very few blastula were observed at this temperature. At other temperatures however, gastrula were definitely noticeable. These gastrula could be seen to dart across the field of the microscope. Blastula could also be seen, with visible cilia and spinning as expected.

The change in the ratio of gastrulating embryos as a function of temperature is graphed in the appendix. A z-statistic for the two populations was performed and the significances at a 95 percent confidence level can also be found in the appendix.

ΔG is obtained by plotting the natural log of the rate of mutation repair as a function of the inverse of temperature in Kelvin, according to the Arrhenius equation (see appendix):
\[ \ln r = \frac{-\Delta G}{R} \cdot \frac{1}{T} \]

\[ \Delta G \text{ (repair enzyme activation energy)} = -R \cdot \text{slope} = -21.6 \pm 0.5 \text{ kJ/mol K} \]

**Discussion**

The results generally appear to be precise but force the original hypothesis to be rethought. With some examination, the data yielded a value with excellent precision. However, as the value for the energy of activation is less than zero, it is impossible to accept this as the energy of activation for the photoreversal enzyme.

The embryo samples were very different across temperatures. Specifically it was noted that the embryos kept at 25°C did not generally survive, even without radiation, and were dramatically malformed compared to cells at other temperatures. The difference between photoreversed and covered populations was also not significant by \(z\)-test, suggesting that, for this sample, photoreversal was not a significant factor in determining gastrulation. For these two reasons, this point was ignored in the Arrhenius plot. The 19°C sample also failed the \(z\)-statistic significance test by a small amount. While the 95 percent confidence level cutoff was 1.96, the \(z\)-statistic for this value was 1.90. Because the shortcoming was small the data point was left in the plot.

Although the final standard error for the calculation of \(\Delta G\) was small, the value is probably not as accurate as the plot would suggest. Since there were only three samples, it is likely that their high linearity represents a statistical anomaly. Certainly, a more careful experiment should be performed with more data points between 4 and 19°C, before this value is accepted.

There are a number of potential sources of error for this experiment. The counting of gastrula and blastula would have been more accurate if more embryos were counted. This could have also led to higher values for the \(z\)-statistic, which would increase the confidence in these three points. There is also some uncertainty in the temperature values, both due to variability in cold room temperatures and to the time between irradiation (conducted at 25°C) and the embryo’s placement in their respective cold rooms.
Most surprising, however, was the positive slope of the Arrhenius plot, yielding a negative value for the energy of activation of the photoreversal enzyme. A catalyst with negative activation energy is self-contradictory since this would imply a transition state with a lower energy than the products and reactants, causing the catalyst to bind irreversibly with the substrates. In any case, this negative activation energy most likely implies a problem in the original model of photoreversal. One satisfying explanation is that this experiment actually measured the activation energy of some tumor suppressor gene. This protein would be responsible for initiating apoptosis when it detected sufficiently serious flaws in the embryonic chromosome. As the temperature was lowered, the less temperature dependent, photoreversal enzyme had a greater chance to operate before the tumor suppressor terminated development. The reason why we would measure the activation of one activation energy and not the other is that, as the activation energy for a given enzyme increases, so does its temperature dependence. The design of this experiment will measure the activation energy of the most temperature dependent protein. Whatever this value represents, it is clear that the photoreversal process is more complicated than was previously thought.

Future experiments would be necessary to address this issue. The best way to determine the actual activation energy for the photoreversal enzyme would be to isolate it in vitro for microcalorimetry studies. It would also be useful to perform the same experiment on sea urchin gametes with a tumor suppressor knockout. This test could support this alternative model for temperature dependent DNA repair.

References


Chart I: Gastrulation Results

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Entering Gastrulation</th>
<th>Mutants</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 Foil</td>
<td>39</td>
<td>16</td>
<td>0.71</td>
</tr>
<tr>
<td>13 Covered</td>
<td>81</td>
<td>32</td>
<td>0.72</td>
</tr>
<tr>
<td>19</td>
<td>88</td>
<td>25</td>
<td>0.78</td>
</tr>
<tr>
<td>25</td>
<td>3</td>
<td>151</td>
<td>0.02</td>
</tr>
<tr>
<td>Temperature</td>
<td>Entering Gastrulation</td>
<td>Mutants</td>
<td>Ratio</td>
</tr>
<tr>
<td>4 With Light</td>
<td>103</td>
<td>10</td>
<td>0.91</td>
</tr>
<tr>
<td>13</td>
<td>62</td>
<td>10</td>
<td>0.86</td>
</tr>
<tr>
<td>19</td>
<td>57</td>
<td>6</td>
<td>0.90</td>
</tr>
<tr>
<td>25</td>
<td>20</td>
<td>358</td>
<td>0.05</td>
</tr>
</tbody>
</table>

\[
\Delta \text{Ratio} = \Delta \text{Ratio} / (1800 \text{s})
\]

<table>
<thead>
<tr>
<th>(1/T)</th>
<th>Ln (k)</th>
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<tbody>
<tr>
<td>3.61E-03</td>
<td>-5.09</td>
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<tr>
<td>3.50E-03</td>
<td>-5.43</td>
</tr>
<tr>
<td>3.42E-03</td>
<td>-5.57</td>
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<td>3.36E-03</td>
<td>-10.89</td>
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**Arrhenius Plot**

\[ y = 2594.3x - 18.471 \]

\[ R^2 = 0.9873 \]

Table 1: \(-\Delta G/R\) is given by the slope of this line
Calculation of $\Delta G$

$\Delta G = -R \text{slope} = -21.6 \text{ kJ/mol K}$

<table>
<thead>
<tr>
<th>P1</th>
<th>P2</th>
<th>$p\bar{\text{bar}}$</th>
<th>s.e.$\left(P1-P2\right)$</th>
<th>$z\text{-stat}$</th>
<th>Significant (95%)</th>
</tr>
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<tbody>
<tr>
<td>0.71</td>
<td>0.91</td>
<td>0.845</td>
<td>0.059</td>
<td>3.18</td>
<td>Yes</td>
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<tr>
<td>0.72</td>
<td>0.86</td>
<td>0.773</td>
<td>0.063</td>
<td>2.10</td>
<td>Yes</td>
</tr>
<tr>
<td>0.78</td>
<td>0.90</td>
<td>0.824</td>
<td>0.060</td>
<td>1.90</td>
<td>No</td>
</tr>
<tr>
<td>0.02</td>
<td>0.05</td>
<td>0.043</td>
<td>0.019</td>
<td>1.40</td>
<td>No</td>
</tr>
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