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# The Chemistry of Signal Transduction in the TetR System

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## Abstract

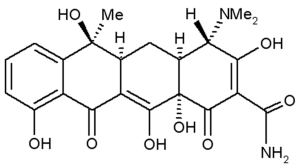
Signal transduction proteins in biological systems must be very flexible to undergo the allosteric changes necessary for their function. It is current practice to investigate the modes of action of these systems by X-ray spectroscopy of the different states trapped as crystals. Unfortunately, the forces acting on the proteins by packing effects may lead to distortions comparable to the changes that occur during the allosteric movements. This makes it questionable as to whether X-ray structures can be used to deduce induction mechanisms. In this work, we show for DNA-binding tetracycline repressor proteins that molecular dynamics simulations offer an interesting alternative for determining the induction state and possible mechanisms switching between them. Based on data sampled for different repressor classes with several force field parameter sets, we show that MD simulations have convincing advantages over the analysis of static structures influenced by crystal packing.

## Introduction

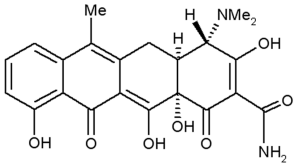
Signal transduction is the mechanism by which biological processes are turned on and off. Organisms use signal-transduction pathways to control their development, react to external stimuli such as heat, cold, excess or lack of nutrients, toxic chemicals etc., but also to regulate cell growth and death. Malfunction of signal-transduction systems can lead, among others, to cancer or autoimmune diseases. The effects of many hereditary diseases can also

be attributed to malfunction in signal-transduction processes. Thus, signal-transduction pathways and processes play a major role in the systems chemistry of living systems because they combine to form complex signaling networks that control the development, metabolism and defense mechanisms of living organs.

Perhaps the structurally and mechanistically best characterized signal-transduction protein is the tetracycline repressor (TetR)[1]. TetR switches an efflux-pump defense mechanism [2] in resistant Gram-negative bacteria when they are subjected to tetracycline antibiotics.




Tetracycline (Tc)




5a,6-Anhydrotetracycline (ATc)

The parent compound tetracycline (Tc, shown above) is a natural product produced by several *Streptomyces* strains. The metabolite 5a,6-anhydrotetracycline (ATc, show above) is not an effective antibiotic but induces TetR approximately 500 times better than Tc [3] and is therefore often used in biological experiments rather than a tetracycline antibiotic.

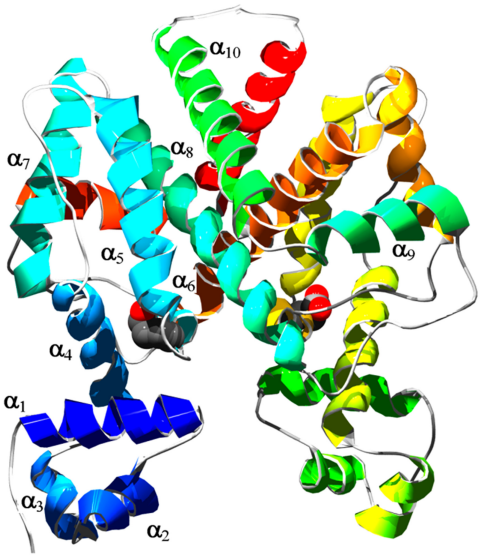



**Figure 1.** Schematic view of the tetracycline repressor as its homodimer. The α-helices of the front monomer are numbered. The DNA-binding heads contain a helix-turn-helix motif and consist of the first three helices.

Apart from the therapeutic use of tetracycline derivatives (doxycycline is the most commonly used tetracycline and a new derivative, tigecycline, was introduced recently), the importance of the tetracycline/TetR system lies in its use as a "gene switch" [4]. TetR can be introduced into both prokaryotes and eukaryotes in order to be able to turn specific genes on and off at will by administering tetracyclines. TetR has thus become an important tool in molecular biology.

The mode of action and mechanism of induction of TetR are therefore important, both for developing more specific TetR switches that can be used in parallel in one organism and also because of the general significance of signal transduction in biological processes. Quite generally, repressor proteins that switch the expression of other proteins on and off bind to the promoter region of a gene and in doing so prevent expression of the encoded protein by blocking access of RNA-polymerase, the enzyme that synthesizes transfer RNA based on the sequence of the gene. Switching occurs when the repressor protein undergoes an allosteric rearrangement that weakens its binding to the promoter, so that the RNA-polymerase can displace it and proceed to transcribe the gene. In bacteria, TetR binds to the promoters belonging to the genes that encode the tetracycline antiporter (TetA), a membrane-bound protein that pumps tetracycline as the complex of its anion with $Mg^{2+}$ out of the cell. TetR, however, also regulates its own expression, so that when TetA has pumped all the tetracycline out of the cell, more TetR is available to turn off the expression of the two proteins once more. Thus, any disadvantages that the bacterium may suffer from the presence of TetA in its cell wall are avoided by the switching mechanism.

The structure of TetR is shown schematically in Figure 1 and its complex with DNA [5] in Figure 2.

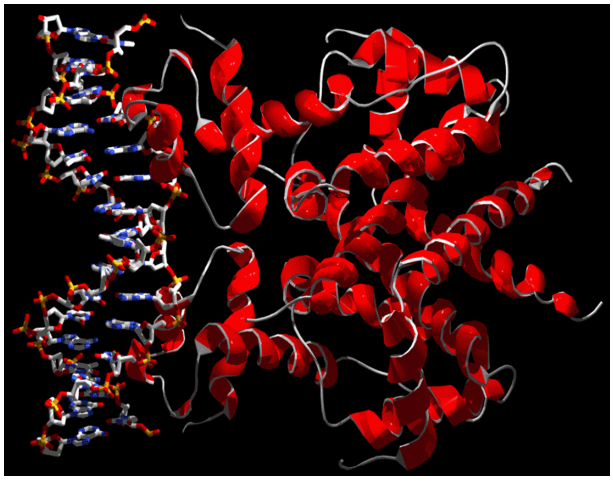


**Figure 2.** The homodimeric tetracycline repressor bound to DNA (structure taken from PDB entry 1QPI [5])

The contact between the protein and DNA occurs via the helices α3, so that the distance between the centers of gravity of the $C_\alpha$ atoms of these two helices (denoted R(α3-α3′) in the following) determines whether the repressor fits into the major groove of the double helix, and therefore how well it binds.

## Molecular Dynamics Simulations

The mechanism of induction of TetR was investigated using molecular dynamics (MD) simulations. In this technique, a classical force field (in this case AMBER Parm94 [6], Gromos87 [7] or OPLS_AA [8]) has been used to simulate the movements of the protein by solving Newton's second law of motion starting from random velocities for the atoms [9]. Because the TetR protein is very flexible, implicit water solvent must be used and long-range electrostatic interactions included using the particle-mesh Ewald (PME) technique [10]. The details of the simulations are given in the original article [11].

The main problem in determining the mechanism of induction, which was thought to occur on a timescale of microseconds to milliseconds, was that the length of time that could be simulated when the original studies were carried out was limited to tens of nanoseconds. Thus, it was impossible to observe the allosteric change on induction in the simulations. However, facile rearrangements of biological macromolecules reveal themselves in the normal vibrations. The normal modes with the lowest frequency and the largest amplitude are usually those that lead to the rearrangement. It is not necessary to calculate the force-constant matrix for the system as the normal modes can be calculated by diagonalizing the mass-weighted covariance matrix obtained from an MD simulation [12].

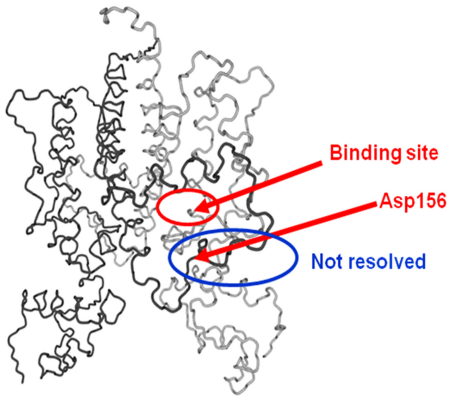



**Figure 3.** $C_\alpha$ trace of the backbone of TetR showing the flexible loop, which is not resolved in most X-ray structures, the tetracycline binding site and the position of Asp156.

Figure 3 shows the main features of the induction mechanism revealed by the lowest-energy normal mode. The so-called flexible loop, indicated by the blue oval in Figure 3, has only recently been resolved in some X-ray structures. The lowest-energy normal mode revealed a very large movement of aspartate 156 away from the tetracycline binding site towards the DNA-binding heads. This movement is the key to the induction mechanism, which is shown schematically in Figure 4.

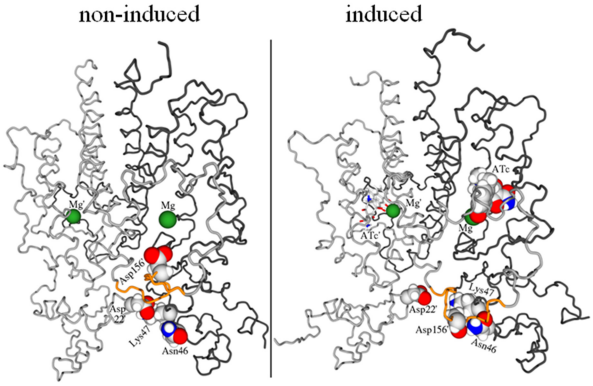



**Figure 4.** Schematic view of the mechanism of induction of TetR by tetracyclines complexed to magnesium. [11] In the non-induced structure (left), Asp156 forms a loose salt bridge to the magnesium ion in the tetracycline binding site and Asp22 forms a tight salt bridge with Lys47 to bind the two DNA-binding heads together. In the induced form (right), ATc displaces Asp156 from the magnesium. The aspartate then migrates towards the DNA-binding heads and forms a new salt bridge with Lys47, thus breaking the one with Asp22. This removes the salt bridge between the two binding heads and allows them to move apart. The flexible loop is highlighted in yellow.

The effect of the inducer (in this case ATc) is to displace Asp156 from the magnesium ion. The aspartate then swings down towards the DNA-binding heads as part of the large movement of the flexible loop observed in the lowest-frequency normal mode. It displaces Lys47 from its salt bridge with Asp22 and thus removes the salt bridge that binds the two DNA-binding heads together.

## The Reverse Phenotype

One of the most fascinating aspects of TetR is that a single mutation is enough to reverse the behavior of the repressor [13]. The reverse phenotype is produced, for instance, by mutating Gly95 in the wild type TetR class BD to glutamate. We will designate this mutant revTetR. RevTetR is induced in the absence of tetracyclines, but not in their presence. Its induction behavior is thus exactly the opposite of the wild type. We have also performed long MD

simulations on this mutant [14] and conclude that the mechanism of induction is exactly the same as that observed for wild type TetR. However, the structure of the revTetR dimer without tetracycline differs significantly from that of the wild type.

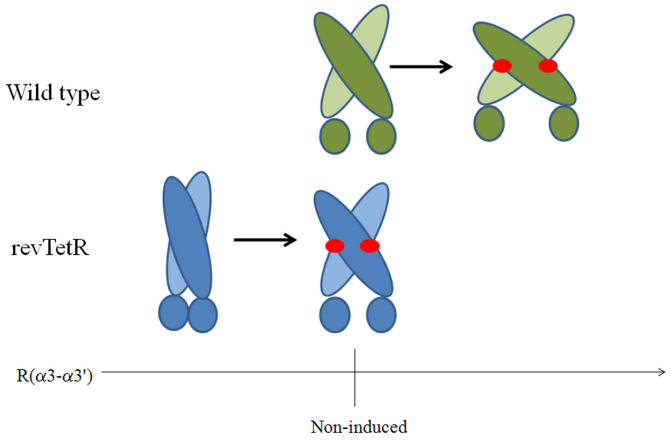



**Figure 5.** Schematic diagram of the mechanism of induction of the TetR wild type (green above) and the reverse phenotype (blue, below). The horizontal axis represents the distance between the DNA-binding heads, which is optimal for binding to DNA at the distance marked "Non-induced". Docked tetracycline molecules are indicated as red ovals.

Figure 5 shows the situation schematically. The R(α3-α3′) distance is too short to bind effectively to DNA in the wild type without tetracycline. The induction movement is the same in the two TetR variants, but in the wild type it extends R(α3-α3′) to a value too large to to bind ideally to DNA, whereas in revTetR it increases R(α3-α3′) to exactly the value needed for optimal binding. Note, however, that revTetR shows signs of denaturing far more easily than the wild type and is stabilized by tetracyclines [15], so that denaturation (and hence induction) may occur in the absence of tetracyclines. Such an effect is too slow to be revealed by the MD simulations.

## Detecting Induction

Experimental determinations of the induction state of TetR are time-consuming and difficult. It would therefore be useful to be able to determine whether a given TetR variant or mutation is induced in the presence of a given inducer. The R(α3-α3′) criterion suggested above may, however, indicate induction in X-ray structures or those taken from MD simulations. In the following, we examine these two possibilities.

## Determining Induction from X-ray Structures

A total of 14 X-ray structures of TetR have been published [16 – 24] with and without a variety of inducers and in one case complexed to DNA. One additional TetR class D structure (2VKE) complexed with Tc and $Co^{2+}$ was published 2007 and shows almost no differences in backbone geometry and ligand position when comparing to 2TRT. Figure 6 shows a plot (in chronological order) of the R(α3-α3′) distances obtained from these structures. There are actually 16 distances because the structures 2NS7 and 2NS8 contain two different TetR dimers in the unit cell. The points in the graph are color coded according to whether TetR is induced or not in the combination found in the X-Ray structure.

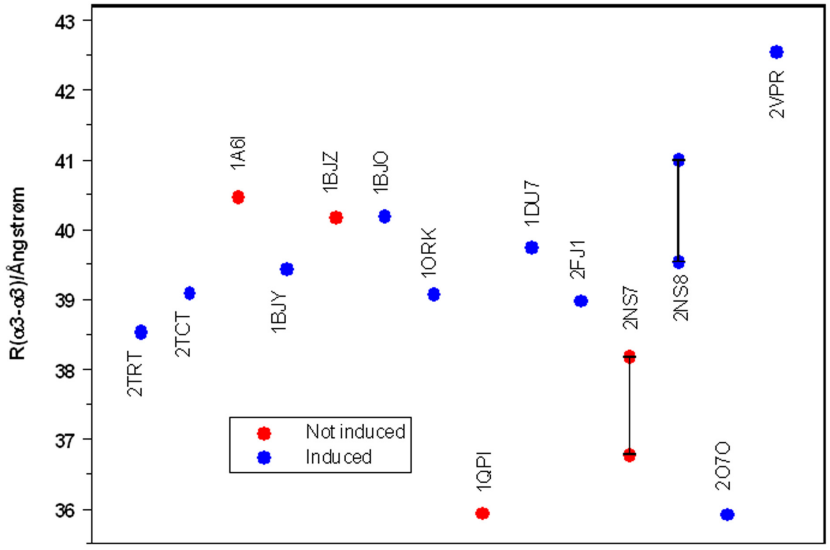



**Figure 6.** Plot of the R(α3-α3′) distances obtained from TetR X-ray structures. The labels for the points indicate the PDB-codes. Two values are given for 2NS7 and 2NS8 because there are two non-equivalent TetR dimers in their unit cells. The points are plotted in the order of their publication.

Figure 6 does not reveal a convincing relationship between R(α3-α3′) and the state of induction of TetR. The definitive R(α3-α3′) value for the non-induced state of TetR is that for 1QPI, which is the X-ray structure in which the TetR dimer is bound to DNA. However, this distance is almost identical to that found in structure 2O7O, a complex with the strong inducer doxycycline. Quite generally, the R(α3-α3′) values from the X-ray structures appear not to be related to the induction state of TetR.

This is not completely surprising. Signal-transduction proteins that switch by undergoing an allosteric change are by their nature very flexible. It is therefore reasonable to expect that the forces needed to switch the conformation are of the same order of magnitude as

crystal-packing forces. The structures of signal-transduction proteins in crystals will therefore be perturbed quite strongly from their solution conformations and cannot be expected to reproduce the induction state of the protein correctly. Note, for instance, that if the first five X-ray structures of TetR had been used to propose a mechanism, we would have concluded that the R(α3-α3′) distance in the non-induced form is larger than in the induced one – the reverse of what we now believe.

## Determining Induction from MD Simulations

Molecular dynamics simulations offer a possible alternative for determining the induction state of TetR. However, for this to be the case, the allosteric rearrangement must take place on a time scale that makes it probable within the length of a simulation, which in our case is 50 – 100 ns. We will show below that, contrary to our expectations, this is the case.

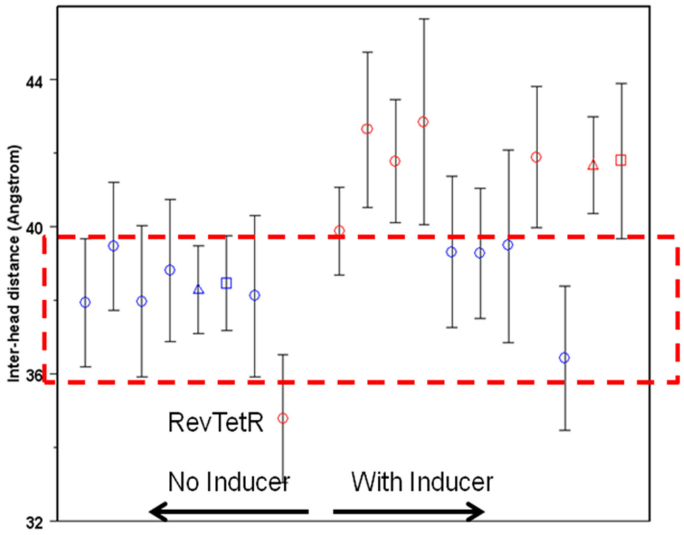



**Figure 7.** Plot of the R(α3-α3′) distances obtained from TetR MD simulations. Red points indicate those that are experimentally induced, blue those that are not. The shapes of the symbols indicate the force field used. Circles are AMBER Parm94, [6] triangles Gromos87 [7] and squares OPLS_AA. [8] The dashed box indicates the limits of R(α3-α3′) for non-induced TetR. The error bars represent ± one standard deviation over the sampling period.

Figure 7 shows the results of 19 simulations, all of which were 50 ns or longer. In contrast to the X-ray results, the R(α3-α3′) distances from the simulations show a clear relationship to the induction state of TetR. All simulations for which R(α3-α3′) falls within the "non-induction window" from approximately 36 – 40 Å represent TetR systems that are found experimentally not to be induced. Those above and below this window are induced.

The differences between some simulations close to the borders are not statistically significant, but nevertheless the simulations provide a remarkably reliable prediction of the induction state.

This result is quite remarkable. MD simulations with three different force fields on a variety of combinations of TetR variants and inducers can reproduce the induction behavior of TetR with far higher reliability than X-ray structures, which are perturbed too much to reflect the induction state correctly. This result even appears to be fairly independent of the force field used. The simulations used three different common force fields, AMBER Parm94, [6] Gromos87 [7] and OPLS_AA [8]. However, many of the simulations did not start in the region of the mean R(α3-α3′) values over the sampling periods. The R(α3-α3′) values shown in Figure 7 are taken from the later stages (typically after 20 ns or longer) of the simulations after TetR has relaxed to its preferred geometry under the conditions of the simulation. This process in itself is of interest.

## Allosteric Rearrangements in MD Simulations

Figure 8 shows a trace of the R(α3-α3′) distance in an AMBER Parm94 simulation of TetR with doxycycline starting from the geometry of the 2O7O X-ray structure. This is the structure that exhibits the same R(α3-α3′) distance as 1QPI, the complex with DNA.

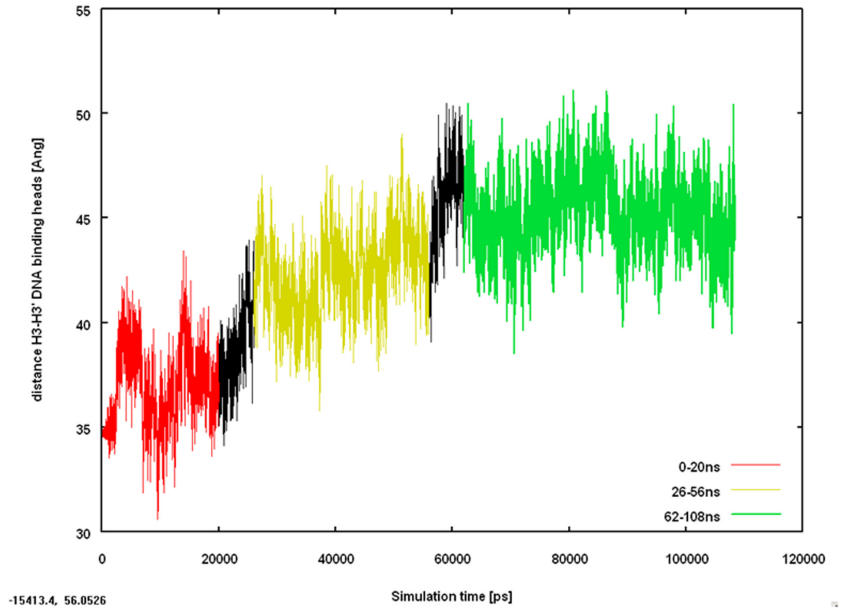



**Figure 8.** Trace of the R(α3-α3') distance against time for a simulation of TetR with two doxycycline molecules starting from the 2O7O X-ray structure.

The red region of the plot from 0 – 20 ns has a mean R(α3-α3′) distance of 36.9 Å, but shows oscillations that appear to increase in amplitude with time. Between 20 and 62 ns, R(α3-α3′) increases steadily to a final value (green region, 62 – 108 ns, mean R(α3-α3′) = 45.3 Å) in which R(α3-α3′) no longer exhibits the strong oscillations seen at the beginning of the simulation. Thus, the simulation remains stable at a non-induced value of R(α3-α3′) for the first 20 ns before undergoing a transition over 40 ns to achieve a final, apparently stable, induced conformation. We emphasize that this process does not necessarily correspond to a real induction event in the biological system, but strictly speaking only to the relaxation of the strained 2O7O X-ray structure. Nevertheless, the fact that the initial conformation remains stable for 20 ns (the complete length of many simulations reported in the current literature) is very significant. This is as far as we are aware the first direct observation in an MD simulation of the pre-equilibrium mechanism of induction [25]. In this mechanism, which has found wide acceptance, the inducer first docks into the non-induced conformation of the repressor to form a metastable complex, which eventually relaxes to the induced conformation of the repressor-inducer complex. In the reverse process, the inducer dissociates from the induced complex to form a metastable, uncomplexed induced conformation of the repressor, which then can relax to the stable non-induced conformation.

## Conclusions

We have shown that MD simulations of 50 – 100 ns are an effective tool for identifying the mechanism of induction of TetR and for detecting its state of induction. These conclusions are presumably also valid for other signal-transduction proteins that vary their binding affinity by an allosteric rearrangement. Perhaps predictably, X-ray structures are not well suited for determining the induction state of signal-transduction proteins because we believe the crystal-packing forces to be of the same order of magnitude as those that cause the allosteric rearrangement.

It is perhaps misleading to discuss "the mechanism of induction". Figure 9 illustrates this point.

Thus, not induction is unique, but rather the fact that signal-transduction proteins can bind so strongly to the promoter and that this bound conformation is so robust to environmental factors such as temperature, pH, ion concentrations etc. that should not be able to cause induction. Induction can be caused by just about any structural change, including denaturation, that lowers the binding affinity. Thus, one and the same signal-transduction protein may be induced in many ways. This is in fact true of TetR, which is induced by tetracyclines in the presence of magnesium, by 5a,6-anhydrotetracycline [26] and the TetR-inducing protein TIP [22] in its absence. In the latter case, a different mechanism of induction is observed to that found with tetracycline-magnesium complexes [27].

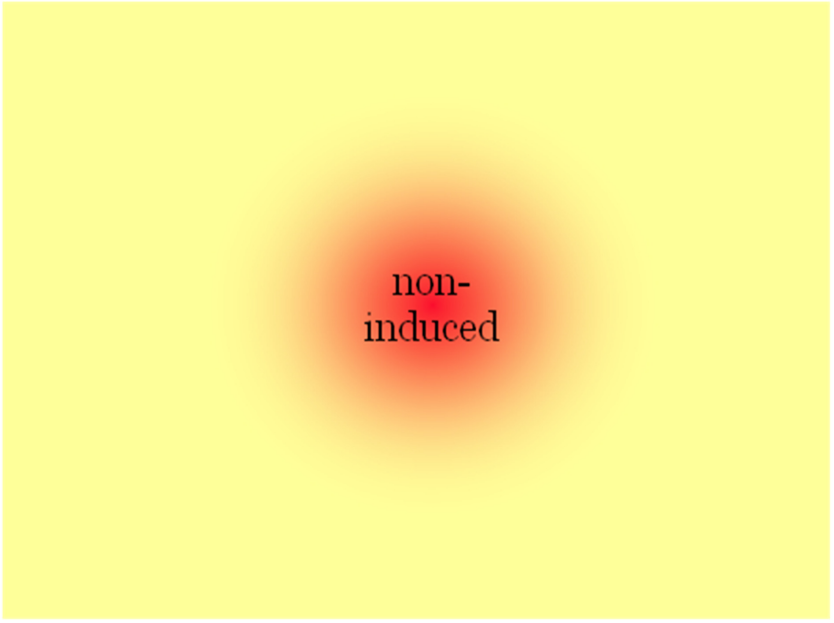



**Figure 9.** Schematic illustration of the induction of a signal-transduction protein. The small, specific red area represents the non-induced protein, which can bind strongly to the promoter. The surrounding yellow area represents induced geometries that do not bind as strongly. Clearly, there are very many ways to distort the protein so that it no longer binds strongly.

## Acknowledgments

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