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Factors influencing protein tyrosine nitration – structure-based predictive models

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Abstract

Models for exploring tyrosine nitration in proteins have been created based on 3D structural features of 20 proteins for which high resolution X-ray crystallographic or NMR data are available and for which nitration of 35 total tyrosines has been experimentally proven under oxidative stress. Factors suggested in previous work to enhance nitration were examined with quantitative structural descriptors. The role of neighboring acidic and basic residues is complex: for the majority of tyrosines that are nitrated the distance to the heteroatom of the closest charged sidechain corresponds to the distance needed for suspected nitrating species to form hydrogen bond bridges between the tyrosine and that charged amino acid. This suggests that such bridges play a very important role in tyrosine nitration. Nitration is generally hindered for tyrosines that are buried and for those tyrosines where there is insufficient space for the nitro group. For *in vitro* nitration, closed environments with nearby heteroatoms or unsaturated centers that can stabilize radicals are somewhat favored. Four quantitative structure-based models, depending on the conditions of nitration, have been developed for predicting site-specific tyrosine nitration. The best model, relevant for both *in vitro* and *in vivo* cases predicts 30 of 35 tyrosine nitrations (positive predictive value) and has a sensitivity of 60/71 (11 false positives).

Keywords

tyrosine nitration; hydrophobic interactions; oxidative stress; tyrosyl radical

Introduction

Investigation of protein tyrosine nitration has intensified over the last two decades leading to a better understanding of the role of this post-translational modification in cellular signaling [1]. Although initially considered to be a marker of oxidative stress, there is a growing body of experimental data suggesting that nitration of tyrosine fulfills the criteria of a signal transducing mechanism [1,2]. For example, tyrosine nitration has been detected under

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physiological conditions in most organ systems and in a number of cellular models. Furthermore, accumulating data supports a strong link between protein tyrosine nitration and the activation of signaling pathways in a variety of cellular responses and pathological conditions, including the cellular response to irradiation, acute and chronic inflammation, graft rejection, chronic hypoxia, tumor vascularization and the microenvironment, atherosclerosis, myocardial infarction, chronic obstructive pulmonary disease, diabetes, Parkinson's disease and Alzheimer's disease [3–20].

Nitration at tyrosine residues occurs both *in vitro* and *in vivo*. Generally, one of the two tyrosine aromatic hydrogens that are ortho with respect to the hydroxyl group is replaced by a nitro group. However, in some instances, nitrotyrosine (nTyr) can further react and replace a second hydrogen atom with another nitro group. Most commonly, tyrosine is nitrated post-translationally in two steps as shown in the scheme below. First, the tyrosine is oxidized to a tyrosyl radical, which in one of its resonance forms, is nitrated in the second step.

Several chemical nitrating species have been implicated in tyrosine nitration including $\bullet\text{NO}$, $\bullet\text{NO}_2$, ONOO^- , $\text{O}_2\bullet^-$, H_2O_2 , NO_2^- , NO_2CO_3^- and $\text{CO}_3\bullet^-$ [21]. NO_2Cl has also been suggested as a contributor; however, its role in tyrosine nitration *in vivo* is highly unlikely [22]. Although most evidence suggests that tyrosine nitration occurs post-translationally, it is formally possible that tyrosine does not have to undergo nitration within the protein. Nitrotyrosine can be transported into cells and then incorporated in proteins during translation [23]. In this case, however, the random incorporation of nitrotyrosine residues in the protein should be observed and this is not the case *in vivo*.

Tyrosine nitration in proteins does not occur randomly. Most proteins contain tyrosine residues (natural abundance: 3.2%) [24], and tyrosine is often surface-exposed in proteins (only 15% of tyrosine residues are at least 95% buried) and should be easily nitrated [1,25]. However, not all exposed tyrosine residues and not all proteins are nitrated. Neither does the abundance of tyrosine residues in a given protein predict whether it is a target for nitration [1,25,26]. This evidence strongly argues that protein tyrosine nitration is a selective process.

Specific amino acid sequences determine the specificity for other post-translational modifications involving tyrosine. For example, the peptide sequence surrounding a tyrosine residue contributes to the substrate specificity of tyrosine kinases [27]. An analysis of the primary sequences of proteins nitrated under similar conditions failed to reveal a specific or unique sequence requirement [26].

Several studies have attempted to determine factors that promote selective tyrosine nitration. It has been reported that tyrosines are more likely to become nitrated when they are in loops [28], which can be expressed as its location with respect to turn-inducing amino acids like glycine and proline [25]. The role of sulfur-containing amino acids in tyrosines nitration has been debated. Some evidence suggests that these amino acids impede tyrosine nitration [26,29] by competing with tyrosine for nitrating species, while others contradict this finding [30], suggesting that sulfur-containing amino acids promote tyrosine nitration. Souza and co-investigators suggest that the presence of acidic residues near the target tyrosine makes it more susceptible to nitration [31]. Lin *et al.* have shown that when glutamic acid 149 is mutated to alanine in cytochrome P450 2B1, a protein known to be nitrated at tyrosine 190, this nitration is substantially reduced [32]. It has also been shown that tyrosine nitration is facilitated by the presence of a nearby basic amino acid [30] and that the hydrophobicity

or hydrophilicity of the environment influences tyrosine nitration [33–35]. Finally, the presence of transition metals seems to encourage nitration of tyrosines [36–39].

Unfortunately, to date there is still no reliable model for predicting tyrosine nitration, as single factors do not satisfactorily explain its selectivity. Other studies have stressed the importance of the protein secondary structure and the local “structural environment” of nitrotyrosine sites [1,25,26]. For example, Gow *et al.* proposed a mechanism defining specificity of tyrosine nitration that requires consideration of the local environment of tyrosine residues within the secondary and tertiary structures of a protein [2].

In this paper we report the first comprehensive and quantitative investigation of protein structural features that influence tyrosine nitration, and in so doing have tested the above anecdotal and qualitative proposals. We have also created an extensive set of additional possible structural factors with potential roles in site-specific tyrosine nitration. Our analysis tested these structural metrics against a training set of known nitrated and (likely) non-nitrated tyrosines; thus, we have built statistical models that can predict tyrosine nitration under different conditions.

Materials and Methods

Data Set

The nitrated tyrosines we have considered in this work are listed in Table 1. This consists of three overlapping sets: proteins that are nitrated *in vitro* by chemical means, proteins that are nitrated *in vivo* by physiological mechanisms, and the union set of All proteins that are nitrated. Thus, results in this work will be referenced to these data sets: “*in vivo*”, “*in vitro*” or “All”. For the *in vivo* and *in vitro* data sets, the positive controls were tyrosines that have been shown experimentally to be nitrated (see Table 1). For these data sets, we used as negative controls tyrosines in those same proteins that were not reported as nitrated. Additional negative controls available for peroxiredoxin I and porcine aconitase were also used in training our models (Table 1). For the All data set, the positive controls were tyrosines used in the two independent data sets. However, because nitration conditions are typically much harsher *in vitro* than *in vivo*, we could not confidently use the *in vivo* negative controls in the All data set, and so used only the *in vitro* negative controls.

Molecular Modeling

Protein structures were obtained from the RCSB Protein Data Bank [51] and manipulations on them were performed with the Sybyl 7.3 molecular modeling program [52]. For each protein in the training set only one chain containing nitrated tyrosines was used for building models. Additional chains, ligands, waters, ions and prosthetic groups were removed. Missing hydrogens were added to the remaining chain using tools in the Sybyl Biopolymer suite.

For each tyrosine examined in this study, three molecular models were built: first, for the wild type (non-nitrated) protein; second, for the tyrosine nitrated at the 3-position (CE1); and third, for the tyrosine nitrated at the 5-position (CE2). To nitrate at the 3- or the 5-position, the appropriate hydrogen atom (HE1 or HE2) was replaced by a nitro group. All models, nitrated or not, were geometry optimized for all atoms within 7 Å of the phenyl oxygen of the tyrosine with the following settings: force field – Tripos; dielectric constant – 6; charges – Gasteiger-Hückel; optimization algorithm – Powell; termination criteria – energy gradient < 0.05 kcal / Å-mol or 5000 iterations.

Some proteins in the training set had multiple subunits (see Table 1). In most cases we did not have definitive information about whether the auxiliary subunits (i.e., not containing the

tyrosine of interest) are present at the time and under the conditions when the nitration occurs (as opposed to the conditions under which the X-ray crystals were grown). For consistency, we used only the chain/subunit containing the nitrated tyrosine for model training, deleting others. However, we recognized that additional subunits could have an impact on tyrosine nitration. So, for the multimeric proteins, we later tested our one-chain training set models on the all-subunit protein molecular models.

Hydropathic Analysis and Computational Titration

A number of our descriptors and structural analysis tools involved hydropathic analysis using the HINT program [53,54]. HINT is a computational model and empirical free energy forcefield based on the partitioning of small molecules between 1-octanol and water, i.e., $\text{LogP}_{\text{o/w}}$. HINT interaction scores are calculated as the total of atom-atom pair-wise interactions; they have been shown to correlate with free energy of association [54]. Computational titration is an application based on HINT that optimizes the placement and orientation of protons so as to have an idealized interaction between the “ligand” and “receptor”. This idealized interaction may include protonation or deprotonation of basic and acidic residues, and or ligand functional groups, respectively [55–58]. For computational titration the protein model was separated into two parts: tyrosine as the pseudo-ligand and the rest of the protein as the pseudo-receptor. The backbone portions of these two fragments are at covalent (bonding) distance to each other and steric repulsion would completely dominate the computed HINT interactions, so the backbone portion of the tyrosine was deleted such that the remainder of the tyrosine, i.e., only its sidechain, was treated as if it were a ligand.

Computational titration was performed on our public web server at <http://hinttools.isbidd.vcu.edu/CT> [59] with the following settings: rotation of amide groups on asparagine and glutamine residues was allowed; on acid residues aspartic and glutamic acids and on C-termini the carboxylate/carboxylic acid groups were titrated and hydrogens in the acid cases were allowed to rotate; histidine was titrated and its ring orientation was checked; lysine and arginine were titrated and for unprotonated lysine the amine rotation was optimized while for unprotonated arginine the hydrogen placement was optimized; for serine, threonine and tyrosine the -XH ($\text{X}=\text{O},\text{S}$) rotation was optimized, while for tyrosine the hydroxyl group was also titrated.

Statistical Model Building

The number of potentially significant descriptors coded for this study was large and many were correlated. Thus, it was impractical to generate models manually and we developed an automated approach with a stochastic algorithm scheme based on ant-colony optimization [60–62], which has been successfully used in constructing QSAR models [63]. In our implementation of ant-colony-based algorithm each descriptor-value pair was assigned some amount of “pheromone”. In each iteration, a fixed number of descriptor-value pairs were picked randomly with a probability proportional to the amount of “pheromone” associated with them. If it was possible to construct a model from these descriptor-value pairs that was better than any other model found so far in this run, then the picked descriptor-value pairs were rewarded with the amount of “pheromone” proportional to the number of cycles needed to find this new model after finding the previous “best” model.

For the largest amount of flexibility in proposing mechanisms, we constructed independent statistical models for three sets of tyrosine nitration data: nitrations that occur *in vivo*, nitrations that occur *in vitro*, and All nitrations (the union set). To avoid constructing over-determined and statistically irrelevant models due to the limited training data available, our algorithm only allowed each model to include a limited number (3 or 4) of descriptor-value

pairs. In analogy to the ant colony model, these descriptor-value pairs are path segments and a model is an entire path of 3 or 4 segments. Each pair had a threshold score value assigned to it. If a calculated descriptor value for a particular tyrosine was greater than its threshold value, then that calculated value was added to a total score for that tyrosine. A model created in this way also included a critical value of the final score, which is, in effect, a metric for ant trail path length. Ideally, tyrosines with a total score over that critical value were nitrated and most having a total score below that value were not.

Results

To probe the many assertions made in the past concerning structural factors important to tyrosine nitration, we created a large number of descriptors (summarized in Table S1 of Supporting Information) that represented these factors in terms of quantitative and testable hypotheses. Throughout this contribution (including Supporting Information), histograms are used to illustrate the responsiveness of nitration to a panel of structural descriptors. In all of these figures, vertical black bars represent tyrosines in the data set(s) that are nitrated and vertical white bars represent those that are not. The y-axes of these diagrams represent the relative fractions of nitrated and non-nitrated tyrosines as a function of the x-axis descriptors. Note that there are many more *not* nitrated tyrosines in the data sets than nitrated tyrosines, so each white, *not* nitrated graph bar represents several times more tyrosines than a similarly sized black bar. Additionally, inset into most of these plots is a horizontal bar at a notable break point that indicates the fraction of tyrosines nitrated (and *not* nitrated) above or below that break point. This indicates an overall usefulness of the descriptor in differentiating the two cases – a “perfect” descriptor would show 100% on both sides of the break point. In the Discussion section, we build multiple-descriptor models that attempt to combine the chemical and structural information from the most successful of the various hypotheses.

The Role of Secondary Structure

A correlation previously observed was that tyrosines are more likely to get nitrated when they are in loops [30]. This is often expressed as being close in sequence to turn-inducing amino acids such as glycine and proline [25]. We accounted for this effect by creating a binary descriptor named *loop* that set a value of 0 for tyrosines in α -helices and β -sheets and 1 for all other tyrosines, where the secondary structure elements were as reported in the protein's PDB coordinate file. As expected, nitration was favored, but not by a particularly significant margin, in loops and disfavored to a similar extent in α -helices, while no trend for tyrosines present in β -sheets is apparent (see Figure 1).

The Role of Sterics

Based on previous reports [31], we expected steric effects to play a major role in tyrosine nitration. We have experimented with many descriptors and found that the best performance in prediction was achieved by *sterics8*, which is the count of heavy atoms of other amino acids within 8 Å of the hydroxyl oxygen of the tyrosine in question. *Sterics8* not only describes how deeply the tyrosine is buried, but also can suggest the morphology of the surface. For tyrosines found on concave surfaces, e.g., in pocket regions, the value of this descriptor is generally greater than that for those on convex surfaces. Both *in vivo* (Figure 2A) and *in vitro* (Figure 2B), nitration was inhibited for buried tyrosines (*sterics8* > 80). *In vivo*, this corresponds to 83 % of nitrated residues at or below *sterics8* = 80, while *in vitro*, nitration was most favored (79 %) for tyrosines when *sterics8* is between 60 and 80, i.e., there are between 60 and 80 heavy atoms within 8 Å of the tyrosine oxygen. The conditions for nitration of tyrosines via chemical means are optimal when the residue is neither buried nor very exposed to the solvent.

Another descriptor used to describe sterics was named *direction_outside*. This is the difference between the distance to the center of the protein from the hydroxyl oxygen (A) and the distance to the center of the protein from the center of the phenyl ring of the tyrosine (B) (see below). The value of this descriptor can range from $-2.78 \text{ \AA}$ for a tyrosine that points directly towards the protein center to $2.78 \text{ \AA}$ for a tyrosine that points completely away from the protein center.

The influence of this descriptor (Figure 2C) was somewhat unexpected, in that we had reasoned that tyrosines with high values of *direction_outside* would be more accessible, and therefore more readily nitrated. This was indeed the case where *direction_outside* was between 1.0 and $2.5 \text{ \AA}$ where 55% of the nitrated (and 43% of *not* nitrated) tyrosines were found. However, there were a few somewhat surprising results. First, *in vivo* and *in vitro* nitrations showed very similar trends (thus, we are illustrating only the All nitrations case). Second, nitration was clearly not favored for tyrosines that pointed directly away from the center of their protein (*direction_outside* $> 2.5 \text{ \AA}$). An explanation could be that as tyrosines contain a hydrophobic benzene ring that can interact more favorably with the protein interior than with water, tyrosines on the protein surface, which are mostly exposed, prefer to tilt and maximize their interactions with the protein. In contrast, tyrosines on the surface, but mostly buried, will try to maximize interactions with water by exposing as much of their hydroxyl group as possible, and thus point away from the protein center. Possible nitration sites of such tyrosines are probably sterically hindered. There is an experimental consideration that must also be considered in this argument – surface residues, such as tyrosines standing up on the surface, generally have less certain coordinates in crystallography due to thermal motion and other factors that lead to disorder. Also interesting was that nitration was strongly favored for tyrosines within the narrow range of *direction_outside* of -1.0 to $-0.5 \text{ \AA}$. These tyrosines are tilted away to a modest degree from the protein surface.

The Roles of Backbone and Polar/Non-polar Sidechains

While it is easier to transmit charge from one location in a protein to another through its backbone [64,65], sidechain atoms near the backbone are less likely to be on the protein surface. To quantitatively describe these competing effects we introduced three descriptors *distance_to_backbone_O*, *distance_to_backbone_N* and *distance_to_backbone* to measure the distance from the hydroxyl oxygen of the target tyrosine to the closest oxygen, nitrogen or any heavy atom of the backbone respectively (with the exception of those of target tyrosine). For the latter descriptor, no clear trends are observed *in vivo* (Figure 3A), although nitration is slightly favored for tyrosines when *distance_to_backbone* is greater than $5 \text{ \AA}$ (50% nitrated), where they are more exposed, more accessible to nitrating species and better able to participate in protein-protein interactions. Somewhat more clear is that *in vitro* nitration (Figure 3B) is favored for tyrosines where *distance_to_backbone* is under $4 \text{ \AA}$ (57

% nitrated), which is consistent with a mechanistic role for charge transfer reactions [66,67], as might be expected for chemically-induced nitration.

It has been previously reported that hydrophobicity and/or hydrophilicity of the surrounding environment influences tyrosine nitration [33–35]. We used three descriptors to help us in assessing the hydrophobicity of the environment: *distance_to_polar_sidechain*, *distance_to_non-polar_sidechain* and *distance_to_neutral_sidechain*. These descriptors measured the shortest distance from the hydroxyl oxygen of tyrosine to the closest heavy (non-hydrogen) atom in the sidechain of polar (arginine, asparagine, aspartic acid, glutamic acid, glutamine, histidine, lysine, serine, threonine and tyrosine), non-polar (alanine, cysteine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan and valine), or neutral (alanine, asparagine, cysteine, glutamine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine) amino acid residues. The most useful descriptor of these three was *distance_to_polar_sidechain*. Proximity of the tyrosine hydroxyl to heavy atoms of polar sidechains had somewhat opposite effects *in vitro* and *in vivo* (see Figure 3C and 3D). *In vivo*, tyrosine nitration was disfavored (65 % not nitrated) when the closest heavy atom belonging to a polar side chain was less than 4 Å away from the tyrosine's hydroxyl oxygen. *In vitro*, nitration was favored (93 % nitrated) when such an atom was less than 5 Å away, again supporting the possible involvement of a charge transfer mechanism for these nitrations. Similarly, *distance_to_neutral_sidechain* (data not shown) indicated that nitration was disfavored for tyrosines when the hydroxyl oxygen was over 4 Å away from the closest heavy atom of the neutral side chain.

The Role of Acidic and Basic Residues

Some have argued that the presence of acidic residues near the target tyrosine makes it more susceptible to nitration [31], while others have shown that tyrosine nitration is facilitated by the presence of a nearby basic amino acid residue [30]. In addition, nitrated tyrosines have been shown to interact favorably with arginine [42,45]. We modeled the effects of nearby basic and acidic amino acids with several descriptors. First, because the mechanism of nitration may involve a tyrosyl radical, we assessed the relative abilities of the tyrosines' environments to support this with *protonation_difference* – the difference in the protein-tyrosine HINT scores between the best scoring computational titration model where the target tyrosine is protonated (neutral) and the best scoring model where it is deprotonated (tyrosinate). The value of this descriptor is greater when proton acceptor groups like deprotonated carboxylates are close to the hydroxyl oxygen of tyrosine and can “accept” the tyrosyl proton, and proton donor groups are far away and cannot stabilize the deprotonated tyrosine by donating their protons. In contrast, the value of *protonation_difference* is lower (more negative) when there are basic amino acids nearby. In neutral nanoenvironments, *protonation_difference* has a value near zero. *Protonation_difference* showed similar behavior with tyrosine nitration for the *in vivo* (Figure 4A) and *in vitro* (Figure 4B) nitration cases. For both *in vitro* and *in vivo* tyrosines, when the value of *protonation_difference* is moderately-to-highly negative, i.e., –25 or less, tyrosines are unlikely (13 % *in vivo*, 14 % *in vitro*) to get nitrated.

We also attempted to describe the influence of acidic and basic amino acids with geometric descriptors that measured the distances from the hydroxyl oxygen of the target tyrosine to the closest atom of another amino acid meeting various criteria. For descriptors *distance_to ASP_O*, *distance_to_GLU_O*, *distance_to_ARG_N*, *distance_to_HIS_N* and *distance_to_LYS_N* (all not shown) the criteria were met by the sidechain heteroatom of the corresponding amino acid. For *distance_to_acidic_sidechain* (Figure 5A) these criteria were met by any heavy atom in a sidechain of an acidic amino acid (aspartic or glutamic acid), and for *distance_to_basic_sidechain* (Figure 5B), by any heavy atom in a sidechain of a

basic amino acid (arginine, histidine or lysine). Because these latter two descriptors exhibited patterns similar to descriptors based on the distances to sidechain heteroatoms of the individual amino acid types, they were used in our further analyses. First, it is clear that when the closest acidic or basic sidechain is 9 Å or more distant, a tyrosine is fairly unlikely to be nitrated, but we had expected these descriptors to be more discriminating. On closer examination, we noted a more interesting effect in the histograms: there are distance-dependent regions of nitration, most with increased probability. The locations at around 4 to 5 Å, 6 to 9 Å and 10 to 12 Å for acidic sidechains (green traces in Figure 5A) and around 3 to 5 Å, 6 to 7 Å, 8 to 9 Å and 10 to 11 Å for basic sidechains (green traces in Figure 5B), roughly correspond to protein geometries that allow various nitrating species to form hydrogen bond bridges between the hydroxyl group of tyrosine and the sidechains of acidic or basic amino acids (see Figure 5C). These observations suggested the creation of a set of meta-descriptors that aggregate these distance-dependent “peaks” of tyrosine nitration probability. This topic will be discussed below in the Discussion.

Interestingly, hydrogen bonding to the tyrosine hydroxyl appears to somewhat hinder *in vivo* nitration (see Supporting Information, Figures S1 and S2), possibly by precluding that tyrosine from interacting with a nitrating species. In summary, tyrosine nitration is definitely impacted by the presence of neighboring acidic and basic residues, but the effect seems to be largely indirect. These residues may play their largest role by stabilizing intermediate states where nitrating agents are attacking the tyrosine.

The Role of Sulfur

While the role of sulfur-containing groups in tyrosine nitration has been debated [1,26,29,30], it does not appear that sulfur atoms immediately in the vicinity of a tyrosine play much of a role in its nitration (see Figure S3), but, in fact, may impede nitration by competing with the nitrating species or by reacting directly with the tyrosyl radical intermediate as suggested by Ischiropoulos [1] and Zhang *et al.* [29].

The Role of Conjugated Ring Systems

The oxidation step that leads to nitration could be the result of oxidation of another residue followed by electron transfer from tyrosine to that residue. For example, pulse radiolysis experiments with lysozyme demonstrated that a tryptophan residue is oxidized first, followed by electron transfer from a tyrosine, which is then nitrated in a subsequent step [68]. Such electron transfer events can occur with rates as high as 10^3 – 10^6 s⁻¹ for peptides and 120–1000 s⁻¹ for proteins [68–70]. Thus, the presence of nearby conjugated ring systems could play a role in tyrosine nitration. Our structure-based analysis (Figure S4) suggests the influence of more than one effect: nearby conjugated rings can both compete with the tyrosine for the oxidizing species while also stabilizing its radical state.

The Role of Ionization States

Having a degree of ionization state flexibility, i.e., acidic and basic residues, in the region surrounding the tyrosine would seem to provide alternative mechanisms for the tyrosine to redistribute charge and/or for radical species to return to their ground state. The descriptor we have used to describe this environment is called *multiplicity_of_states* and is the number of protonation state ensembles considered by computational titration [55–59] for the tyrosine and its neighborhood. The higher this number, the more acids, bases and/or alternate ionization state schemes are available for the region. For counting ionization states, tyrosine/tyrosinate counts as 2, an ammonium/amine, e.g., lysine, counts as 2, a carboxylate/carboxylic acid, e.g., aspartic and glutamic acid, counts as 3 (ionized, or protonated at either oxygen), etc. [55–59]. The total number of available states is the product of these. Overall, most environments have a modest number of states (≤ 6); *in vivo*, 83 % of nitrations occur in

this region with half of these when there are 6 states (Figure 6A). *In vitro*, 64 % of nitrations occur in the absence of other (than the tyrosine itself) ionizable residues with the remainder in environments with between 6 and 36 states (Figure 6B).

The Role of Hydropathic Interactions

Several additional descriptors were derived from the HINT forcefield model, which is designed to empirically estimate hydropathic interactions and the free energy of interaction between species [54,71,72]. As described in Materials and Methods, the side chains of wild type or nitrated tyrosines were modeled as ligands and scored in their surrounding protein as if it was a “receptor”. First, we calculated *WT_score* – the HINT interaction score between the sidechain of the tyrosine and the rest of the protein. Its value is large when the tyrosine interacts favorably with the rest of the protein, i.e., has intramolecular hydrogen bonds, hydrophobic interactions, etc. Previous work has shown that 515 HINT score units are approximately 1 kcal mol⁻¹ [53,54]. In simple terms, tyrosines that interact favorably with the rest of the protein would not likely be nitrated because many of these favorable interactions would likely be lost. *In vivo*, nitration is favored (54 % nitrated vs. 28 % not nitrated) for tyrosines when the value of *WT_score* is negative (Figure 7A). *In vitro*, nitration is only slightly favored (86 % vs. 69% not nitrated) when *WT_score* is greater than -100 (Figure 7B). The *nitrated_score* descriptor is similar except that it assesses the interactions of the putative nitrated tyrosine side chain with the rest of the protein. *In vivo* (Figure 7C), nitration is slightly favored when *nitrated_score* is negative (71 % nitrated vs. 56 % not nitrated) and disfavored when it is positive (44 % not nitrated vs. 29 % nitrated). *In vitro* (Figure 7D), the effect is somewhat the opposite: nitration is favored (64 %) when *nitrated_score* > -100 HINT units and disfavored (65 %) when *nitrated_score* < -100 HINT units. (Further analyses of these effects are illustrated in Supporting Information Figure S5.) The *in vivo* data is somewhat counterintuitive. We suggest that because *in vivo* tyrosine nitration often occurs concomitantly with a change in inter-protein interactions, a nitrated tyrosine with significant favorable intra-protein interactions will not likely participate in inter-protein interactions.

It should be noted that with all structure-based descriptors, particularly those that are assessing the environment post-nitration, a key assumption is that the nitration does *not* result in a major structural rearrangement of the protein. Also, it continues to be evident that *in vivo* and *in vitro* nitrations are favored under somewhat different conditions. An interesting inference about structure can be drawn, however, from the *in vivo* nitration data. As expected, a number of tyrosines with poor (negative) *WT_score* values (Figure 7A) are nitrated; however, it is somewhat counterintuitive that tyrosines with poor *nitrated_score* values (Figure 7C) would be nitrated. As this indicates that these nitrotyrosines would be unstable in their environment, we can infer that some structure rearrangement must be simultaneously occurring to alleviate unfavorable interactions.

Discussion

In the results above, we have probed most, if not all, of the various hypotheses concerning nitration of tyrosines in proteins. This work used three-dimensional structural data rather than sequence to examine the biochemical and related factors involved. While others have examined similar nitration factors through detailed biostatistics approaches using sequence analyses [30,31,73], or with largely qualitative 3D analyses [25,30], this is the first detailed and quantitative investigation of the relationship between nanoenvironment and tyrosine nitration. Clearly, all of the structural effects we described, along with the relevant descriptors we constructed to probe them, are individually not competent to predict nitration. However, nearly all of the effects we probed *are* telling a piece of the story. The wide range of suggestions made in the literature regarding what structural effects promote nitration is, at

a minimum, indicative of a possibility for multiple nitration mechanisms. In this discussion we will describe our efforts to construct meta-descriptors that can represent the interesting distance-dependent behavior noted in our examination of the role of neighboring acidic and basic residues, describe the building of predictive multi-variable models for tyrosine nitration that incorporate information gleaned from several descriptors, and return to the issue of tyrosine nitration in multiple subunit proteins.

Meta-descriptors Describing Acidic or Basic Environment

We observed a very interesting distance-dependent effect in Figures 5A and 5B. There were peaks of high nitration probability very closely corresponding to the chemically-meaningful bridging distances that would be expected (Figure 5C) from various chemical entities that have been implicated in tyrosine nitration. To superimpose these regions with increased incidence of tyrosine nitration, we introduced two meta-descriptors:

relative_distance_to_closest_negative_amino_acid_heteroatom to aggregate the tyrosine nitration regions created by the presence of heteroatoms from acidic aspartate and glutamate amino acid sidechains and *relative_distance_to_closest_positive_amino_acid_heteroatom* to aggregate the tyrosine nitration regions created by the presence of heteroatoms from basic arginine, histidine and lysine amino acid sidechains. Both of these descriptors were calculated in the same way in three steps: 1) we obtained the distances from the hydroxyl oxygen of the tyrosine to the closest relevant sidechain heteroatom of each amino acid of interest [74], e.g., as in descriptors like *distance_to_Asp_O*; 2) for each descriptor thus calculated, there were one or two values in the All nitration dataset around which there was an significant number of nitrated cases. The descriptors were then adjusted by subtracting the closest center value (Figures 5A and 5B); 3) the resulting descriptor with the smallest absolute value (among those calculated for each residue type) is recorded as final descriptor, i.e., either *relative_distance_to_closest_negative_amino_acid_heteroatom* or *relative_distance_to_closest_positive_amino_acid_heteroatom*. The histograms for these two resulting descriptors both had a region near 0, where tyrosine nitration was very probable; however, this region was somewhat more prominent for acidic amino acids than that for basic amino acids (see Figure 8).

Building Multiple Descriptor Models

Most of the descriptors we used in the investigations above do give some useful information with respect to nitration or non-nitration of tyrosines. However, none of them on their own provided more than a 2:1 discrimination enhancement between nitrated and *not* nitrated cases. One approach to further our understanding is to develop multiple variable regression models that combine the information from several descriptors into a single predictive mathematical model. In addition to the descriptors we investigated while testing the various hypotheses of tyrosine nitration above, we also developed a number of other structural descriptors. The large number of potential descriptors necessitated a two-step process in building models. Our first automated attempts used a liberal limit in the number of descriptors allowed in the generated models. As expected, many models were thus created with high-scoring solutions. The resulting top models were seemingly very close to each other in value, but were likely statistically over-determined and often lacked chemical and/or structural interpretability. However, from these successful preliminary models we obtained the working subset of descriptors (Table S1 in Supporting Information) that were partially validated by the preliminary model building, and that could, more importantly, be easily understood in terms of structure.

While there are several statistical methods for building multiple-descriptor models, in this work we have used a novel model building technique based on ant colony-optimization (see Materials and Methods) to derive more universally predictive models. This method is more

compatible with the diverse nature of the descriptors and training set data we have available, i.e., that nitration/non-nitration is both binary and “fuzzy”, in that the definition of nitration is with respect to a varying threshold. Concomitantly, as seen above for most descriptors, nitration is not a smooth function of the descriptor values. In the ant-colony optimization method the values of the particular descriptors for each tyrosine are compared to threshold values; if that parameter exceeds the threshold value for a tyrosine, a specific score reward or penalty is assessed. Tyrosines achieving the highest scores are thus presumed by these models to have the highest likelihood of being nitrated. Because of the limited number of “positive” data points, i.e., verified experimental tyrosine nitration, in the training sets, we restricted our models to including only three or four independent descriptors.

Even with the reduced descriptor set, unrestricted model building was informative, but a very large fraction of the models reported descriptors that: did not apply to the entire data set, e.g., *distance_to_LYS_sidechain*, when some of the nitrated cases did not actually have neighboring lysines; contained pairs of obviously (or sometimes less obviously) correlated descriptors, such as both *distance_to_polar_sidechain* and *distance_to_basic_sidechain*; or contained obviously mutually exclusive descriptor pairs, such as both *alpha* and *loop*. We modified our approach to take advantage of the meta-descriptors that indicated the relative distances to negative and positive amino acid heteroatoms. These inherently incorporate multiple effects and are more universal in scope. To assist in the automated model building, we further modified their definition and created two additional meta-descriptors *abs_relative_distance_to_closest_negative_amino_acid_heteroatom* and *abs_relative_distance_to_closest_positive_amino_acid_heteroatom*, which are simply the absolute values of their parent meta-descriptors. However, with the latter, two rules are required: to define the start and end regions of increased nitration probability. Because these descriptors both encompass multiple effects and their use mitigates the risk of correlated descriptors encoding similar information, we ultimately forced our model builder to use both of them.

Three-descriptor models were built for each of the data sets: *in vivo*, *in vitro* and All nitrations. An additional four-descriptor model was built for All nitrations. Four-descriptor models were not built for the component *in vivo* and *in vitro* datasets because we felt that there were insufficient data to build statistically valid models. Table 2 lists the model data for the four independent models. The models based on three and four descriptors are illustrated in Figure 9. The specific results with respect to the positive controls in the models are set out in Table 3. The three-descriptor and the four-descriptor models seemed to do a quite reasonable job in sorting the known nitration cases with the four-descriptor model (for all cases) doing a significantly better job than the three-descriptor model for this dataset.

In all models, except the *in vivo* model, the *abs_relative_distance_to_closest_negative_amino_acid_heteroatom* meta-descriptor was more useful than the *abs_relative_distance_to_closest_positive_amino_acid_heteroatom* meta-descriptor (see Table 2, score fraction). The former descriptor was associated with a larger acceptable range and/or bigger penalty. All four models used different descriptors to supplement the two meta-descriptors (Table 2). The three-descriptor *in vivo* model favored nitration when the closest hydroxyl oxygen of another neutral amino acid was over 10.1 Å away from the hydroxyl oxygen of the target tyrosine. The three-descriptor *in vitro* model favored nitration when the closest heavy atom of neutral side chain was within 3.91 Å of the hydroxyl oxygen of the target tyrosine. This was the only model where the additional descriptor carried more weight (score fraction) than both the meta-descriptors. The three-descriptor and the four-descriptor models for all tyrosine nitrations used different additional descriptors: in the three-descriptor model, nitration was favored when there were 75 or fewer heavy atoms within 8 Å of the target tyrosine’s hydroxyl oxygen (*sterics8*), while in

the four-descriptor model nitration for tyrosines was favored when their hydroxyl oxygens were within 3.54 Å of the backbone (*distance_to_backbone*) and did not have their interactions with other amino acids deteriorate by more than 75.1 HINT units (*nitration_gain*). No sulfur-related descriptor made it into any of these models. That is not to say sulfur has no effect on tyrosine nitration. Sulfur atoms less than 9 Å from the tyrosine's hydroxyl oxygen lower the probability of *in vivo* nitration significantly. However, this condition is seldom met, so it does not lend itself to being used in universal predictive models that are based on a small number of descriptors and limited data.

Molecular models of best-case and worst-case scenarios are presented in Figure 10. The nitration of Tyr198 of actin is modeled in Figures 10A and 10B. All of the factors in the four-descriptor model are met in this case and indeed it received the highest score in the model. In contrast, Tyr79 of *E. Coli* ribonucleotide reductase protein R2 received the lowest score in the four-descriptor model suggesting that it is very unlikely to be nitrated. An overall success rate of our models is presented in Table 4. For the four-descriptor model of all nitrations, our model predicted 30/35 or 86% of the known-to-be-nitrated cases in our training set. Interestingly, most false negatives predicted by these models were predicted by more than one model. For example, all four models missed the nitration of Tyr122 in the *E. Coli* ribonucleotide reductase protein R2. The reason for this may be the proximity of Tyr122 to a center containing two iron atoms that can potentially produce much reactive species and promote tyrosine nitration. The effects of metal atoms were not considered in our computational models.

Another significant point is that the training sets are biased. While the cases we have modeled as “nitrated” are well studied and documented, the tyrosine cases that we have modeled as “not nitrated” are considerably less so. It is quite possible that one or more of these “not nitrated” tyrosines are, in fact, nitrated, but the experiments to determine this have probably not been performed. Thus, the false positive rate from these models may be lower than we have reported.

Nitration of proteins with multiple subunits

One of our assumptions in these analyses was that the structural factors contributing to nitration were localized on the subunit containing the tyrosine. Thus, we modeled the nitration and calculated descriptors based only on that subunit and ignored other subunits that may have been present in the crystallographic (PDB) data file for the protein. The reasons for this assumption were two-fold: first, it is possible and maybe even likely that nitration occurs when proteins are in different conformation or association states from those preserved in their crystal structures; second, we felt that, in this way, we would have a much more consistent and less ambiguous approach to training the models. To validate this assumption we tested the models trained on monomer/single chain representations on the full protein (all subunit) models of IκBα in complex with NF-κB (Tyr181, 1ikn), bovine ribonuclease A (Tyr115, 1jvt), dihydropyrimidinase-related protein 1 (Tyr316, 1kcx), *E. Coli* ribonucleotide reductase protein R2 (Tyr122, 1rib), clathrin adaptor protein complex 1 (Tyr574, 1w63), and triosephosphate isomerase (Tyr67, 2jk2) – the six proteins for which we truncated the protein model to a single subunit in training. Of these six tyrosines, four were predicted correctly by our training model and two were not (Table 3). Also, although it was not included in the training set, we applied our models to Tyr327 in the tetramerization domain of p53, which we have examined previously [45]. This latter test is, thus, an external validation of the model.

The results of these tests are summarized in Table 5. For the six tyrosines in the original data set, this reexamination with all subunits correctly predicted nitration of only two, neither of which was incorrectly modeled previously. This failure is actually an interesting result as it

suggests that at least some of these nitrations do take place while the proteins are monomeric or in an exposed conformation different from the occluded conformations suggested by the full protein's X-ray crystal structures. We can test this assertion with Tyr327 in the tetramerization domain of p53. This nitration can occur reversibly *in vivo* and we have shown that it probably occurs while p53 is in the monomer form [45]. While the structure of the tetramerization domain of p53 has been determined many times [75–78], it has, by experimental necessity, always been crystallized at a concentration high enough for tetramerization to occur. Because the tetramerization domain of p53 is flexible, different conformations are likely assumed by the interfacial residues between the monomer and tetramer forms. We applied the trained models described above on p53 as a monomer, dimer and tetramer (Table 5), but, in accord with experimental evidence [45], only the *in vivo* model on the monomer structure predicted the nitration of Tyr327.

Conclusions

We have developed predictive models for the nitration of tyrosines under oxidative stress, either biologically or chemically. These models correlated experimentally known cases of nitration with detailed three-dimensional structural data for their proteins – particularly with respect to the nanoenvironment of the tyrosine. The statistical analysis in this work is complicated by the nature of the data set: the number of experimentally verified nitrated cases is not large and more than a little bit vague due to different experimental nitrating conditions producing different results – muscle glycogen phosphorylase b nitration has been observed in three different sets of tyrosines in cells from rabbits [79], rats [80] and mice [81]. For the most part, the negative controls are even less certain, being largely tyrosines in the same proteins (as the positive controls) that have not been “observed” to be nitrated. Thus, there are many more negative than positive controls in our training set. Nonetheless, our models were remarkably accurate (> 75%) in terms of identifying the true positives (see Table 4). We were less successful in rejecting false positives, especially for the *in vivo* cases where the difference between nitration and non-nitration may hinge upon a few rather subtle structural features. We believe that a larger data set would enable more accurate and predictive models for this phenomenon, and will continue to enhance this model as additional data becomes available.

While it has previously been noted that acidic or basic residues near a tyrosine are critical factors in its nitration [30,31], a particularly interesting observation in this work was the near-perfect match between the distance dependence for these residues, typified by our meta-descriptors *relative_distance_to_closest_negative_amino_acid_heteroatom* and *relative_distance_to_closest_positive_amino_acid_heteroatom*, and the bridging distances that would be anticipated for insertion of various nitrating agents. Further work may reveal additional mechanistic details that can be examined experimentally. When differences were observed between *in vivo* and *in vitro* nitration behavior, they could generally be explained with a hypothesis that, *in vivo*, open, unobstructed environments are preferred (as further suggested by the multiple chains results), and that chemically-induced *in vitro* nitrations favor more closed environments with nearby heteroatoms or unsaturated centers that can stabilize radicals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Probability of tyrosine nitration for tyrosines found in α -helices, β -sheets and loops for All cases (*in vivo* and *in vitro* combined).

Figure 2.

The influence of descriptors probing steric effects on tyrosine nitration. A) nitration as a function of *sterics8* (number of non-hydrogen atoms within 8 Å of the tyrosine's hydroxyl oxygen) for *in vivo* cases; B) nitration as a function of *sterics8* for *in vitro* cases; C) nitration as a function of *direction_outside* (see text for specific definition) for All nitration cases (combined *in vivo* and *in vitro*).

Figure 3.

The influence of two descriptors probing the role of backbone and polar/non-polar sidechains on tyrosine nitration. A) Nitration as a function of *distance_to_backbone*, which measures the distance from the hydroxyl oxygen of the tyrosine to the closest heavy backbone atom of another amino acid *in vivo* cases; nitration as a function of *distance_to_backbone* for *in vitro* cases; C) nitration as a function of *distance_to_polar_sidechain* for *in vivo* cases; D) nitration as a function of *distance_to_polar_sidechain* for *in vitro* cases.

Figure 4.

The influence of a descriptor probing the role of acidic and basic residues on tyrosine nitration. A) nitration as a function of *protonation_difference* for *in vivo* cases; (B) nitration as a function of *protonation_difference* for *in vitro* cases.

Figure 5.

Influence of descriptors probing the role of acidic and basic residues on tyrosine nitration. A) nitration as a function of *distance_to_acidic_sidechain*, which measures the distance from the hydroxyl oxygen of the target tyrosine to the closest heavy atom of a sidechain of an acidic amino acid (Asp or Glu), for All cases. B) nitration as a function of *distance_to_basic_sidechain*, which measures the distance from the hydroxyl oxygen of the target tyrosine to the closest heavy atom of a sidechain of a basic amino acid (Arg, His or Lys), for All cases; C) scheme illustrating species and “sizes” of agents implicated in tyrosine nitrations and their geometries in possible intermediates. The lengths are the calculated optimal atom-atom distances for the minimum energy conformations of the depicted species. The green profiles superposed on the histograms in panels A and B illustrate “peaks” at distances where nitration occurs (not necessarily distances of enhanced nitration probability.) These peaks roughly correspond to many of the bridging lengths shown in panel C.

Figure 6.

Influence of a descriptor probing the role of ionization states on tyrosine nitration. A) nitration as a function of *multiplicity_of_states*, which is the number of possible protonation states for the tyrosine and its close environment, for *in vivo* cases; B) nitration as a function of *multiplicity_of_states* for *in vitro* cases. The minimum value for this descriptor is two because the tyrosine itself has two states: protonated/deprotonated.

Figure 7.

Influence of descriptors probing the role of hydrophobic interactions on tyrosine nitration. A) nitration as a function of *WT_score*, which is the HINT score between the tyrosine and the rest of the protein in HINT score units, for *in vivo* cases; B) nitration as a function of *WT_score* for *in vitro* cases; C) nitration as a function of *nitrated_score*, which is the HINT score between the putatively nitrated tyrosine and the rest of the protein in HINT score units, for *in vivo* cases; D) nitration as a function of *nitrated_score* for *in vitro* cases.

Figure 8.

Creation of meta-descriptors for distances to acidic and basic sidechains. See text for further description. A) nitration as a function of *relative_distance_to_closest_negative_amino_acid_heteroatom*, which is the difference between the distance from the hydroxyl oxygen of the target tyrosine to the sidechain oxygen of Asp or Glu and the distance that is optimal for nitration, for All cases; B) nitration as a function of *relative_distance_to_closest_positive_amino_acid_heteroatom*, which is the difference between the distance from the hydroxyl oxygen of the target tyrosine to the sidechain nitrogen of Arg, His or Lys and the distance that is optimal for nitration, for All cases.

Figure 9.

Multiple descriptor models for tyrosine nitration. A) model for the *in vivo* data set with three variables; B) model for the *in vitro* data set with three variables; C) model for the All data set with three variables; D) model with four variables for the All data set. False negatives (where true nitration was not predicted) are marked with the PDB codes and residue IDs from those protein structures.

Figure 10.

Molecular models illustrating tyrosine nitration. A) Tyr198 of actin (pdb 1j6z) is experimentally known to be nitrated and received the highest score in the four-descriptor model for All nitrations cases. Notes: 1-the environment is basic and computational titration suggests that the optimal ionization state is tyrosyl with a hydrogen bond to the backbone, 2-the closest positive heteroatom of a basic sidechain is on Arg196, 3-the closest negative heteroatom of an acidic sidechain is on Glu253; B) model of actin with nitrated tyrosine nTyr198. Note: 4-the nitro group of nTyr198 is likely directed into the solvent and interacting with (not shown) water molecules; C) Tyr79 of *E. Coli* ribonucleotide reductase protein R2 (pdb 1rib) is not known to be experimentally nitrated and received the lowest score in the four-descriptor model for All nitrations cases. Notes: 5-the closest negative heteroatom of an acidic sidechain is on Glu283, 6-the closest positive heteroatom of a basic sidechain is on Arg149, 7-the presence of a nearby hydroxyl group (Ser211) is an additional unfavorable factor; D) model of ribonucleotide reductase protein R2 with nitrated tyrosine nTyr79. Notes: 8-purple contours illustrate changes in hydrophobic interactions due to nitration with a reduction in hydrophobic interactions between the tyrosine and the rest of the protein, 9-red contours indicate a reduction in hydrogen bonding and acid-base interactions as well as steric clashes between the tyrosine and the rest of the protein.

Table 1

Proteins and residues used in tyrosine nitration study.

Protein	PDB	Nitrated Tyrosines	Tyrosines Used as Non-Nitrated Controls	Refs.
<i>In vivo</i> nitrations				
Muscle creatine kinase	1i0e	14, 20	39, 82, 125, 140, 173, 174, 279	40
Actin	1j6z	91, 198, 240	53, 69, 133, 143, 166, 169, 188, 218, 279, 294, 306, 337, 362	41
Dihydropyrimidinase-related protein 1	1kcx	316 ^a	32, 36, 75, 135, 145, 167, 170, 174, 182, 251, 290, 336, 395, 431, 479	30
IκBα (monomeric)	1ikn	181 ^a	195, 248, 251, 254, 289	42
Calcineurin	1m63	224	113, 119, 124, 132, 140, 159, 170, 175, 258, 260, 262, 288, 291, 311, 315, 324, 341	30
Mitochondrial creatine kinase	1qk1	274	9, 15, 34, 77, 95, 115, 120, 168, 169, 354	30
Clathrin adaptor protein complex 1	1w63	574 ^a	6, 70, 72, 76, 121, 136, 229, 276, 277, 300, 328, 333, 361, 405, 421, 425, 455, 524, 526, 566	30
Kelch-like ECH-associated protein 1	1u6d	345, 491, 537	329, 334, 341, 375, 396, 426, 443, 473, 490, 520, 525, 567, 572, 584	43
<i>E. Coli</i> ribonucleotide reductase protein R2	1rib	122 ^a , 273, 289	2, 28, 33, 62, 79, 156, 157, 166, 194, 209, 307, 310	44
Muscle glycogen phosphorylase	2amv	113, 161	51, 52, 83, 84, 185, 203, 226, 233, 262, 280, 297, 374, 404, 472, 511, 524, 548, 553, 587, 613, 726, 731, 732, 777, 780, 791, 820	40
14-3-3 β	2bq0	84	21, 50, 106, 120, 127, 130, 151, 180, 181, 213	30
P53 (tetramerization domain) ^b	2j0c	327	None	45
Triosephosphate isomerase	2jk2	67 ^a , 208	47, 164	30
Brain-type creatine kinase, B chain	3b6r	269	39, 68, 82, 100, 125, 173, 174, 279	30
Fructose-1,6-bisphosphate aldolase	3bv4	173, 203	58, 84, 137, 213, 222, 243, 301, 327, 342	30, 43
<i>In vitro</i> nitrations				
P53 (DNA-binding domain)	2fej	107	103, 126, 163, 205, 220, 234, 236	45
Bovine ribonuclease A	1jvt	115 ^a	25, 73, 76, 92, 97	25
Bovine SOD Cu Zn	1e9q	108	none	46
Peroxiredoxin I	2rii	None	116	46
Actin	1j6z	53, 198, 240, 362	69, 91, 133, 143, 166, 169, 188, 218, 279, 294, 306, 337	44
<i>E. Coli</i> ribonucleotide reductase protein R2	1rib	62, 122 ^a	2, 28, 33, 79, 156, 157, 166, 194, 209, 273, 289, 307, 310	47
Muscle creatine kinase	1i0e	82	14, 20, 39, 125, 140, 173, 174, 279	43
Bacteriorhodopsin	1brd	26	43, 57, 79, 83, 147, 150, 185	48
Porcine aconitase	1boj	None	43, 60, 74, 96, 136, 178, 206, 223, 268, 274, 301, 306	49
GST-1	2h8a	92	18, 115, 120, 137, 145	50
Lysozyme	2zxs	20, 23	53	25

^aThese tyrosine environments were represented in model training by single subunits although there are potentially interacting residues in other subunits of that protein.

^bExperimental evidence suggests that p53 is nitrated in its monomeric form. This protein/residue was not used in training, but only in testing the model.

Table 2

Model data for multiple descriptor tyrosine nitration predictions.

Data Set	Model		Descriptor	Threshold Value ^b	Reward or Penalty ^c	Score fraction ^d
	Descriptors	Quality ^a				
In vivo	3	0.779	abs_relative_distance_to_closest_negative_amino_acid_heteroatom	0.220	-3	0.333
			abs_relative_distance_to_closest_positive_amino_acid_heteroatom	0.450	-4	0.444
			distance_to_sidechain_SER_THR_TYR_O	10.1	2	0.222
In vitro	3	0.828	abs_relative_distance_to_closest_negative_amino_acid_heteroatom	1.02	-2	0.286
			abs_relative_distance_to_closest_positive_amino_acid_heteroatom	0.463	-2	0.286
			distance_to_neutral_sidechain	3.91	-3	0.429
All	3	0.809	abs_relative_distance_to_closest_negative_amino_acid_heteroatom	1.02	-4	0.444
			abs_relative_distance_to_closest_positive_amino_acid_heteroatom	0.452	-3	0.333
			sterics8	75.5	-2	0.222
	4	0.845	abs_relative_distance_to_closest_negative_amino_acid_heteroatom	0.599	-7	0.333
			abs_relative_distance_to_closest_positive_amino_acid_heteroatom	0.434	-6	0.286
			distance_to_backbone	3.54	-5	0.238
			nitration_gain	-75.1	3	0.143

^aQuality score of model: $Model\ Quality = \sqrt{\frac{TP}{TP+FN} * \frac{TN}{TN+FP}}$, where TP=true positives, FP=false positives, TN=true negatives and FN=false negatives.

^bIf descriptor value exceeds the threshold, the ^creward or penalty will be assessed to the model.

^dScore fraction represents the relative significance of that descriptor to the overall model.

Table 3

Model predictions for all nitrated tyrosines from the training set (monomeric proteins). Filled circles are tyrosines predicted to be nitrated (true positives). Empty circles are tyrosines not predicted to be nitrated (false negatives).

PDB Code	Nitrated Tyrosine	Three-Descriptor Model Predictions			Four-Descriptor Model Predictions	
		<i>In Vivo</i>	<i>In Vitro</i>	All	All	All
1brd	26	////	•	•	•	•
	108	////	•	•	•	•
1i0e	14	•	////	•	•	•
	20	•	////	•	•	•
	82	////	•	•	•	•
1ikn	181	•	////	•	•	•
1j6z	53	////	•	•	•	•
	91	•	////	•	•	•
	198	•	•	•	•	•
	240	•	○	○	○	○
1jvt	362	////	•	•	•	•
	115	////	•	•	•	•
1kcx	316	•	////	•	•	•
1m63	224	•	////	•	•	•
1qk1	274	•	////	•	•	•
1rib	62	////	•	•	•	•
	122	○	○	○	○	○
	273	•	////	•	•	•
	289	•	////	•	•	•

PDB Code	Nitrated Tyrosine	Three-Descriptor Model Predictions			Four-Descriptor Model Predictions
		<i>In Vivo</i>	<i>In Vitro</i>	All	
1u6d	345	•		•	•
	491	•		•	•
	537	○		•	•
1w63	574	○		○	○
2amv	113	•		•	•
	161	•		•	•
2bq0	84	•		•	•
2fej	107		•	•	•
2h8a	92		•	•	•
2jk2	67	•		•	•
	208	○		○	○
2zxs	20		•	•	•
	23		○	○	•
3b6r	269	•		•	•
3bv4	173	•		○	•
	203	•		○	○

Table 4

Performance of multiple descriptor models. Note that in all training sets the number of tyrosines not nitrated far exceeded the number of tyrosines that were, i.e., there are many more negatives than positives in the data set.

	3-Descriptor Models			4-Descriptor Model
	<i>In vivo</i>	<i>In vivo</i>	All	All
TRUE positives	20	11	27	30
TRUE negatives	123	62	56	55
FALSE positives	46	9	10	11
FALSE negatives	4	3	8	5

Table 5

Evaluation of nitration cases with multiple subunits. Filled circles are tyrosines predicted to be nitrated (true positives). Empty circles are tyrosines not predicted to be nitrated (false negatives).

PDB Code	Nitrated Tyrosine	Protein Form	Three-Descriptor Model Predictions			Four-Descriptor Model Predictions	
			<i>In Vivo</i>	<i>In Vitro</i>	All	All	All
1ikn	181	Complex with p65/p50	○		○	○	○
1jvt	115	Dimer		●	●	●	●
1kcx	316	Dimer	○		○	○	○
1rib	122	Dimer	○	○	○	○	○
1w63	574	Heterodimer	○		○	○	○
2jk2	67	Dimer	●		●	●	●
2j0z	327	Monomer	●		○	○	○
		Dimmer	○		○	○	○
		Tetramer	○		○	○	○