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(54) **Title:** METHOD AND COMPUTER PROGRAM FOR DETERMINING OR ALTERING A COFACTOR SPECIFICITY OF A TARGET ENZYME

Fig. 2
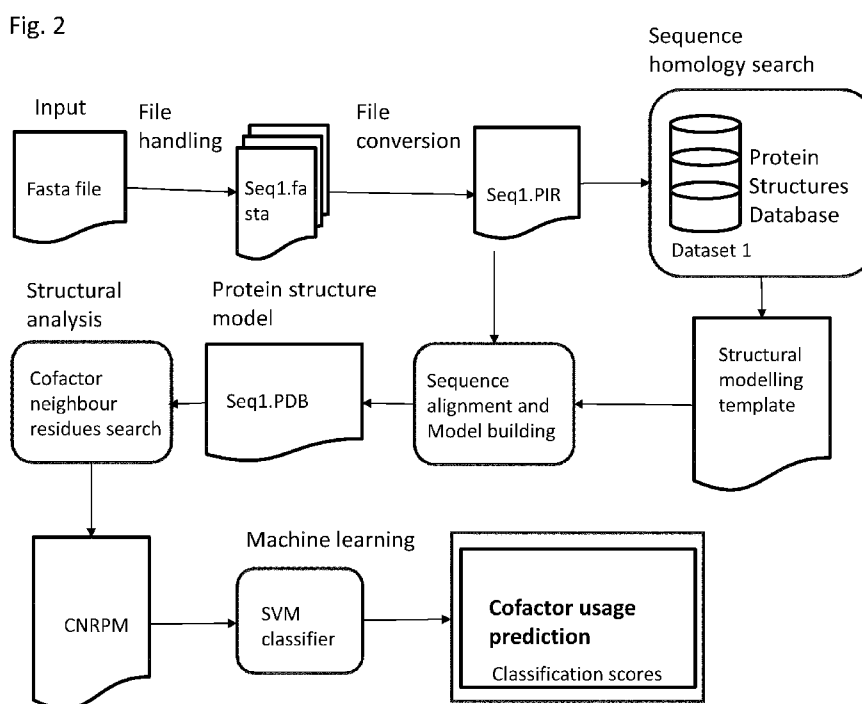
(57) **Abstract:** The invention relates to a method and a computer program for determining a cofactor specificity of a target enzyme, wherein the target enzyme is expected to use one of a first cofactor and a second cofactor based on an amino-acid sequence of the target enzyme, and/or for determining an amino-acid sequence of a target enzyme variant, wherein the variant is characterized by a cofactor specificity differing from that of the target enzyme, the method comprising at least the steps of: providing an atomic structure for each of both cofactors, wherein each atomic structure comprises cofactor atoms, and wherein cofactor atoms in the atomic structures that are located at the same corresponding locations in both atomic structures are selected; providing the amino-acid sequence of the target enzyme; determining an estimated target enzyme-cofactor structure comprising information on a spatial structure of the target enzyme bound to one of the cofactors; generating an interaction matrix for the target enzyme-cofactor structure, wherein the interaction

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matrix comprises entries relating the selected cofactor atoms to surrounding amino-acid residues of the target enzyme; determining a cofactor specificity of the target enzyme by providing the interaction matrix, particularly the entries of the interaction matrix, to a trained classifier that is configured to classify the cofactor specificity of the target enzyme based on the provided interaction matrix to either the first or the second cofactor.

Method and computer program for determining or altering a cofactor specificity of a target enzyme

Specification

5   The invention relates for determining a cofactor specificity of a target enzyme, wherein the target enzyme is expected to use one of a first cofactor and a second cofactor based on an amino-acid sequence of the target enzyme, and/or for determining an amino-acid sequence of a target enzyme variant, wherein the variant is characterized by a cofactor specificity differing from that of the target enzyme.

10   Systems biology foundations broadly rely upon well performed gene annotations. In this post-genomic era, given the large and exponentially growing amount of sequences being characterized, experimental determination of a protein's function is becoming unfeasible, due to its cost and time consumption. Current methodologies are based on pairwise sequence alignment and search for sequence homology to

15   perform protein function annotation. However, the usage of such approaches in annotation pipelines tend to continuously propagate annotation errors across all sequenced organisms due to the attribution of outdated and unspecific functions to new annotated genes, impairing the discovery of new gene functions. Despite their usefulness and relevance, such methodologies fail in capturing essential information

20   hidden in dissimilar areas of different sequences, such as cofactor specificity, which gravely impairs the understanding of an organism's metabolism.

Cofactors act in enzymatic reactions as redox carries and are important mediators for energy transfer in the cell. The lack of accuracy in determining cofactor usage in numerous genes severely affects, for example, genome-scale metabolic model

25   reconstruction, as well as metabolic engineering and strain design endeavours, due to the potential identification of misleading reactions.

Nicotinamide adenine dinucleotide (NAD(H)) and nicotinamide adenine dinucleotide phosphate (NADP(H)), are the most wildly used cofactors in cell metabolism. These structurally similar molecules act as functional group transfer agents, being therefore

30   consumed at the same rate of substrate consumption. Moreover, the uncertainty of their usage in metabolic reactions has a major impact in metabolic engineering applications, affecting both predictions and strain design results. When correctly characterized, enzyme modification by thorough structure redesign for cofactor

specificity change can be undertaken, enabling the efficient processing of multiple desired biocatalytic transformations.

NAD(H) and NADP(H) are functionally equivalent cofactors used for storage and exchange of electrons in catalytic reactions. These cofactors are used by the majority

5   of oxidoreductases, the largest class in Enzyme Commission. The only difference between these two molecules is a phosphate group in the adenine moiety, located on the opposite side from the chemically active nicotinamide moiety. Despite their apparent similarity, enzymes that use these cofactors tend to be specific for only one of them, enabling pathway regulation and chemical driving force maintenance by the

10   cells, through heavy regulation of the levels of oxidized and reduced metabolic pools of NAD(P)(H).

Despite the efforts in identifying the cofactor specificity, very few studies go beyond pinpointing the specific residues Arginine and Aspartate and only for the phosphate moiety area. The best example is a study performed using ketol-acid

15   reductoisomerases that showed that the presence of acidic residues at conserved phosphate binding positions are potential candidates of enzymes preferring NAD(H).

Another problem is the fact that most studies are based on data often composed by small datasets or specific enzyme sub-classes, which can bias the results due to their sequence similarity, and are regularly characterized using visual interpretation

20   or selection of positive cofactor change mutations.

Moreover, one of the most relevant even greater challenge is not only to determine the cofactor specificity of an unknown enzyme, but also to alter the cofactor specificity of enzyme and thus opening the gate for designing biosynthetic pathways, such as the case of nicotinamide adenine dinucleotide (NAD(H)) and nicotinamide

25   adenine dinucleotide phosphate (NADP(H)) pathways.

Due to the structural similarity between NAD(H) and NADP(H), with their only difference residing in the presence of a phosphate group in the vicinity of the 2' hydroxyl of the adenosine ribose in NADP(H), specificity mechanisms are difficult to characterize, hindering rational approaches for performing NAD(P)(H) cofactor

30   specificity reversal or specificity switching.

An object of the present invention is to provide a method and a computer program for determining a cofactor specificity of a target enzyme, wherein the target enzyme is expected to use one of a first cofactor and a second cofactor based on an amino-acid sequence of the target enzyme, particularly wherein the amino-acid sequence is

35   devoid of 2D- or 3D-structural enzyme information. Another aspect of the invention is

to provide a method and computer program for determining an amino-acid sequence of a target enzyme variant, wherein the enzyme variant is characterized by a cofactor specificity differing from that of the target enzyme, wherein the cofactor specificity of the enzyme variant is particularly switched from the first cofactor to the second cofactor. These objects are achieved by the device having the features of claim 1.

Advantageous embodiments are described in the subclaims.

According to claim 1 the method comprising at least the steps of:

    i)   providing particularly an information on an atomic structure for each of
          both cofactors, wherein each atomic structure comprises cofactor atoms,
          and wherein cofactor atoms in the atomic structures that are located at
          the same corresponding locations in both atomic structures are selected,
    ii)   providing the amino-acid sequence of the target enzyme,
    iii) determining an estimated target enzyme-cofactor structure comprising
          information on a spatial structure of the target enzyme bound to one of
          the cofactors,
    iv)   generating an interaction matrix for the target enzyme-cofactor structure,
          wherein the interaction matrix comprises entries relating the selected
          cofactor atoms to surrounding amino-acid residues of the target enzyme,
          particularly wherein for each selected cofactor atom, entries are
          generated in the interaction matrix that comprise the counts of each
          amino-acid residue of the target enzyme within a predefined distance to
          the selected cofactor atom,
    v)   determining, particularly by means of a higher probability score, a
          cofactor specificity of the target enzyme by providing the interaction
          matrix, particularly the entries of the interaction matrix, to a trained
          classifier, particularly a trained support vector machine that is configured
          to classify the cofactor specificity of the target enzyme based on the
          provided interaction matrix to either the first or the second cofactor.

It is noted that the sequence of steps i) to v) and also sequence steps of the subclaims do not have to be carried out according to their numbering or listed sequence. Moreover, separate sequence steps can be executed simultaneously or be comprised in a single step or action. Particularly steps comprising the provision of

features can be merged and be reduced to practice by for example providing the respective features mixed or in combination.

Ad item i) The provision of the atomic structure particularly comprises the retrieval of an information about the atomic structure of the cofactor. The term "atomic structure of the cofactor" particularly refers to the geometrical and spatial relative position of atoms in the cofactor to each other.

The first and the second cofactor particularly differ in their atomic structure in at least one atom, i.e. the atomic structure of one of the cofactors has at least one atom that is not present in the other cofactor. This can be by addition or replacement.

The selected cofactor atoms particularly reflect essentially the atoms common in both cofactors. Atoms in excess are not selected.

Therefore, independent of the cofactor the number of selected atoms is the same, even though the cofactors might not have the same number of atoms. In other words, the selected atoms form a common cofactor devoid of structural features characteristic for the first or the second cofactor.

The atomic structure (and more specifically the information on the atomic structure), can be provided or retrieved for example by a particularly electronic database and stored in a data storage for further processing

The atomic structure can be provided in a common data format for storing and read-out the atomic structures. Moreover, the atomic structure can be provided in combination with other information such as for example in combination with the target enzyme-cofactor structure. It is explicitly noted, that it is not necessary to provide the atomic structure in a separate processing step. It is within the scope of claim 1 that the atomic structure is provided by any means.

Ad step ii) the provision of the amino-acid sequence of the target enzyme particularly refers to the provision or retrieval of information on the amino acid sequence of the target enzyme. In analogy to step i) the information can be stored in an electronic data format and processed accordingly.

The amino acid sequence can provide a numbering of the amino acids such that the amino-acid sequence can be determined by providing number in combination with the kind code of the amino acid. Each amino acid therefore can be associated with a number indicating its unique position in the sequence.

Ad step iii) determining an estimated target enzyme-cofactor structure comprising information on a spatial structure of the target enzyme to one of the cofactors.

The term "bound" in the context of the enzyme and the cofactor particularly also refers to a state of interaction of the enzyme with the cofactor, when the enzyme uses said cofactor, and thus a generic interaction configuration is adopted by the enzyme and the cofactor. Therefore, the term "bound" is to be understood in a broad sense throughout the specification.

The target enzyme-cofactor structure in analogy to the atomic structure of the cofactor comprises the relative positions and particularly orientations of the atoms of the cofactor as well as the amino acids of the target enzyme.

The target enzyme-cofactor structure can be provided, determined or stored in an appropriate data format.

It is noted that the target enzyme-cofactor structure particularly provides information about the positions of the cofactor atoms, including the selected cofactor atoms, with respect to the amino acid residues of the enzyme. Therefore, it is possible to determine distances between the cofactor atoms and amino-acids and other spatial relations between the cofactor and the amino acid residues of the enzyme.

The term "amino acid residue" particularly refers to an amino acid comprised by the enzyme and can particularly addressed by means of an index in the amino acid sequence of the enzyme.

While on some cases the target enzyme-cofactor structure might be known or otherwise inferred, in other cases the target enzyme-cofactor structure has to be determined, for example by a homology search method. The latter is typically the case, when the cofactor specificity of the enzyme is unknown.

In case the cofactor specificity is to be altered or switched from the first cofactor to the second cofactor (or vice versa), the target enzyme-cofactor structure is often known regarding only one of the cofactors, e.g. the first cofactor. As in subsequent steps particularly only the selected cofactor atoms are of importance, knowing the target enzyme-cofactor structure for one cofactor only, e.g. the first cofactor is suffices for proceeding with the next method steps.

It is further noted that the atomic structure of the cofactor might be provided (cf. step i)) only or exclusively together with, e.g. bound to the target enzyme. The same holds true for the provision of the amino-acid sequence of the target enzyme (cf. step ii)).

Ad step iv) generating an interaction matrix for and particularly from the target enzyme-cofactor structure, wherein the interaction matrix comprises entries relating

the selected cofactor atoms to surrounding amino-acid residues of the target enzyme, particularly wherein for each selected cofactor atom of the selected cofactor atoms, entries are generated in the interaction matrix that comprise counts of each amino-acid residue or amino-acid residue type of the target enzyme within a predefined distance to the selected cofactor atom.

The interaction matrix is also referred to as the cofactor neighbor residue profile matrix or CNRPM. The CNRPM can be in generated in a form of a look-up table or any other format that is suited to relate selected cofactor atoms with the surrounding amino acid residues of the target enzyme. In the context of the current specification these formats are considered equivalent and within the meaning of the term "matrix", as they can serve the same purpose.

Entries in the CNRPM particularly consist of a single value that provides the information of the surrounding amino-acids.

According to one embodiment for each cofactor atom M entries are provided, wherein equals to the number of particularly naturally occurring amino acids, i.e. typically 20, or amino acids that are comprised in the enzyme.

Thus, for N selected cofactor atoms and M amino acids, a total of N x M entries are generated. Each entry relating the nth selected cofactor atom with the mth amino acid

Said n^{th} x m^{th} entry comprises for example the number of Lysines with in the surrounding of mth selected cofactor atom, which can be for example a carbon atom.

It is noted that for the purpose of unambiguity, the cofactor atoms, particularly the selected cofactor atoms, can be associated with an index allowing an identification of the cofactor atom in the atomic structure of the cofactor.

The term "counts" particularly refers to the number of occurrences, e.g. the frequency, the specific amino acid is found in the surrounding.

The term "surrounding" particularly refers to an atomic scale surrounding, i.e. in the order of Angstroms.

As the number of selected cofactor atoms is independent of the first and second cofactor, the interaction matrix particularly does not comprise cofactor atoms solely present in one of the two cofactors.

Moreover, the number of entries and particularly the dimension of the interaction matrix is independent of the first or second cofactor and is the same for both cofactors.

This allows for a machine learning method, such as the classifier, particularly the support vector machine to process to be trained with the same number of features for the first and second cofactor.

According to one embodiment of the invention, the predefined distance corresponds to a radius of less than 12A, more particularly less than 6A. "A" stands for Angstrom.

According to this embodiment all amino-acid residues that are within the predefined distance of at least one of the selected cofactor atoms are counted in the respective entry of the CNRPM.

In step v) a cofactor specificity of the target enzyme is determined by providing the interaction matrix, particularly the entries of the interaction matrix, to a trained support vector machine that is configured to classify the cofactor specificity of the target enzyme based on the provided interaction matrix to either the first or the second cofactor.

The classifier, particularly the support vector machine is particularly a machine learning method that during a supervised training phase identifies a hyperplane that separates features from each other such that a classifier is built.

The classifier is trained in a supervised manner with datasets of target enzymes having a known cofactor specificity, particularly a known target enzyme-cofactor structure, more particularly a known associated-interaction matrix, which can be determined from the target enzyme-cofactor structure.

The features for training and for evaluation provided to the classifier are particularly the entries of interaction matrix.

A suitable classifier in form of a support vector machine, can be based for example on the scikit-learn library for python [3].

After training the classifier, particularly the trained support vector machine is used for classifying interaction matrices associated or derived from to a target enzyme-cofactor structure. The classification is for example done by means of a higher probability score output by the classifier for one of the two cofactors.

It is noted that the interaction matrix does not comprise specific structural features of the cofactors as only the selected cofactor atoms are comprised in the interaction matrix.

Thus, even if the target enzyme-cofactor structure is specific to the first or second cofactor the interaction matrix does not comprise these structural differentiating features.

This allows using a molecular structure template of a known enzyme-cofactor structure and adapt said template to a target enzyme having unknown cofactor specificity or an unknown target enzyme-cofactor structure without introducing or at least with minimizing a bias in the interaction matrix that is used for classification.

5   Thus, the support vector machine classifies the cofactor specificity of a given target enzyme by means of the interaction matrix associated to the target enzyme and the selected cofactor atoms.

Therefore, special attention should be paid at the generation step of the target-enzyme-cofactor structure in case it is unknown.

10   The method according to the invention therefore allows determining and particularly predicting the cofactor specificity of a target enzyme, wherein only the amino-acid sequence of the target enzyme and the atomic structure of the cofactor is provided.

The target enzyme-cofactor structure can be determined by means of a homology search and/or a modelling method.

15   According to an embodiment of the invention, the trained classifier provides for each selected cofactor atom and for each cofactor and for each amino acid a feature weight indicative for a cofactor specificity strength of the amino acid.

The cofactor specificity strength and the feature weight is a statistically determined value derived from a training set for the classifier, particularly wherein a higher

20   feature weight for an amino acid indicates that the said amino acid has a supposedly large influence on the cofactor specificity, wherein a lower feature weight indicates a small influence on cofactor specificity.

According to another embodiment of the invention, particularly if the target enzyme-cofactor structure is unknown, the following steps are executed for determining the

25   estimated target enzyme-cofactor structure:
      –   performing a homology search with the amino-acid sequence of the target enzyme in a protein structure database comprising information on molecular structures of enzymes bound to the first or the second cofactor,
      –   assigning the molecular structure comprising the enzyme that exhibits a

30   highest degree of homology to the amino-acid sequence of the target enzyme as a molecular structure template,
      –   from the molecular structure template determining the target enzyme-cofactor structure for the amino-acid sequence of the target enzyme bound to the cofactor particularly by aligning particularly modelling the amino-acid

35   sequence of the target enzyme to the molecular structure template.

A homology search can be performed by using homology models. Such models are for example created using Modeller [7], where sequence similarity search for template selection is performed using the Smith-Waterman local alignment [8], [9] in a local database composed by structures from the Protein Data Bank (PDB) bound to one of the cofactors, such as for example NAD(P)(H). Structural similarity evidencing a suitable template is particularly assume when two enzymes share an amino-acid sequence identity above 25%, i.e. more than 25% of the amino-acid residues are identical.

The atomic structure of the cofactor is particularly allocated in the aligned amino-acid sequence by allowing Modeller to transfer these molecules from the molecular structure template to the modelled structure, which is considered the target enzyme-cofactor structure.

Thus, according to this embodiment the target enzyme-cofactor structure estimated from the molecular structure template might comprise the cofactor bound to the target enzyme for which the target enzyme might not be specific. This issue is particularly resolved by generating the interaction matrix from the selected cofactor atoms only and corrected by determining the cofactor specificity of the target enzyme with the classifier, particularly the support vector machine based on the interaction matrix.

According to another embodiment of the invention, the classifier, particularly the support vector machine is trained by

- providing particularly a protein structure database, the protein structure database comprising information on molecular structures of a plurality of enzymes, wherein each molecular structure represents an enzyme bound to the first or the second cofactor, particularly wherein each molecular structure is provided in the same format as the target enzyme-cofactor structure, particularly wherein the atomic structure of the bound cofactor in relation to the amino-acid residues of the respective enzyme is comprised by the molecular structure.

- for each molecular structure, generating the interaction matrix particularly according to step iv), wherein each interaction matrix is associated to the respective cofactor of the molecular structure,

- training the classifier, particularly the support vector machine with the interaction matrices so that the classifier, particularly the support vector

machine is trained to classify the cofactor specificity for the first or the second cofactor of an enzyme based on the entries of the interaction matrix.

The molecular structures can be provided by the PDB.

Once the classifier is trained, the classifier particularly estimates a probability for the target enzyme to be specific for the first and/or second cofactor. If probability for the first and/or second cofactor is greater than a threshold value it is classified to be specific for this cofactor.

According to another embodiment of the invention, the trained classifier, particularly the support vector machine provides a cofactor specificity probability for the first and/or the second cofactor for classifying the cofactor specificity of the target enzyme, wherein if said cofactor specificity probability exceeds a predefined threshold value, the target enzyme is classified to be specific to the cofactor with the probability exceeding said threshold value.

The cofactor specificity probability particularly assumes values between 0 and 1, wherein the threshold value for the probability is particularly larger than 0.5, more particularly larger than 0.8, even more particularly larger than 0.9.

According to another embodiment of the invention, the amino-acid sequence of the target enzyme having the cofactor specificity switched from the first cofactor to the second cofactor is determined by the steps of:

a)   particularly providing a target enzyme cofactor structure with a target enzyme being specific to the first cofactor,

b)   prior to step v), replacing at least one amino-acid in the interaction matrix,

c)   determining whether the cofactor specificity determined in step v) is switched from the first cofactor to the second cofactor,

d)   particularly repeating the steps b) to c), particularly until the cofactor specificity determined in step v) is switched from the first cofactor to the second cofactor.

Step a) this step can be achieved already in step iii). This target enzyme-cofactor structure serves as the basis for modifications on the enzyme, wherein form the target enzyme-cofactor structure the interaction matrix is generated according to step iv). Once the interaction matrix is established, at least one amino-acid residue of the

amino-acid sequence of the target enzyme is changed. This can be done directly in the interaction matrix according to step b) and translated to the amino-acid sequence of the target enzyme resulting in an amino-acid sequence for an enzyme variant.

It is noted that changing one amino-acid residue of the amino-acid sequence of the target enzyme might result in multiple changes in the interaction matrix, in case said amino-acid residue is comprised in multiple entries of the interaction matrix.

The altered interaction matrix is then provided to the trained classifier, such that a cofactor specificity of the enzyme variant is determined. In case the specificity remains unaltered another or more amino-acids can be changed until the cofactor specificity of the associated enzyme variant is witched form the first cofactor to the second cofactor.

This embodiment allows for determining an amino-acid sequence of an enzyme variant having a switched cofactor specificity with respect to the original target enzyme.

According to another embodiment of the invention, for each of the two cofactors and for each of the selected cofactor atoms and for each amino-acid, particularly when it is within the predefined distance of the cofactor atom, a feature weight indicative of a cofactor specificity strength is determined or provided, particularly wherein the classifier determines and/or provides the feature weights after being trained, particularly wherein said feature weights are provided in form of a computer readable look-up table.

The plurality of feature weights associated to the first or second cofactor and to the respective cofactor atom is also referred to as impact matrix in the current specification.

This embodiment allows identification of amino-acids for each selected cofactor atom that have a large influence on cofactor specificity. The identification is achieved by means of comparably (with respect to estimated feature weights of other amino acids) large feature weight.

It is noted that the feature weights are determined for amino acids in the surrounding, particularly within the predefined distance of the respective cofactor atom. The feature weights are particularly not connected to a specific enzyme or amino acid

sequence but represent a generalized property of the cofactor specificity resulting from the training of the classifier, particularly the support vector machine.

The feature weight can particularly assume values between 0 and 1 or scaled to this range.

According to another embodiment of the invention, a maximum impact matrix is determined or provided relating for each cofactor, each selected cofactor atom to the amino-acid having the largest feature weight for the respective cofactor specificity, particularly wherein the maximum impact matrix is determined and/or provided by the trained classifier, particularly the trained support vector machine, particularly wherein the maximum impact matrix stored as a computer readable look-up table.

The maximum impact matrix is particularly generated after training of the classifier is completed, wherein the maximum impact matrix does not necessarily need to be generated each time the trained classifier determines the cofactor specificity of the target enzyme.

The maximum impact matrix can be sorted according to the feature weight, particularly such that the selected cofactor atoms associated with the amino acids with the largest feature weights are in the first rows or columns.

According to another embodiment of the invention, the amino-acid sequence of the target enzyme, i.e. the enzyme variant, having the cofactor specificity switched from the first cofactor to the second cofactor is determined by the steps of:

- before the interaction matrix is provided to the classifier, particularly the support vector machine in step v), replacing M amino-acid residues in the interaction matrix corresponding to the amino-acid residues of the target enzyme with the M largest feature weights for the first cofactor, with the corresponding M amino-acid residues for the same cofactor atoms having the largest feature weight for the second cofactor, wherein M is a natural number, particularly wherein the maximum impact matrix is used for looking up M amino acids for the first and second cofactor,

- particularly providing the such altered interaction matrix to the classifier, particularly the support vector machine as the interaction matrix in step v),

–   after step v), determining whether the specificity of the enzyme variant associated to the interaction matrix is switched with respect to the target enzyme.

The M amino-acids having the M largest feature weights of the corresponding cofactor atom can be for example looked up from the maximum impact matrix.

Particularly from the maximum impact matrix the cofactors having the amino acids with the largest feature weight for the first cofactor are identified and the amino acids for the same cofactor atoms are identified (particularly from the maximum impact matrix) that have the highest feature weight for the cofactor specificity for the second cofactor.

Then, the M amino-acids with the M highest feature weights for the first cofactor are replaced with the amino acids (for the same cofactor atom) having the highest feature weight for the second cofactor.

This embodiment particularly uses the maximum impact matrix, however it is possible to do so also by selecting the respective amino acids from feature weights determined in the impact matrix.

M is particularly between 1 and 10.

This embodiment provides a deterministic approach for replacing the amino-acids.

According to another embodiment of the invention, starting from an initial value for M, particularly M =1, the amino acid residue having the M highest feature weights for the first cofactor are replaced by the corresponding amino acids for the second cofactor, wherein M is incremented by one or more in case the specificity is not switched, until the cofactor specificity of the target enzyme is switched or a predefined maximum value for M is reached.

As elaborated above, the switching can be established by the classifier that determines the cofactor specificity particularly based on a cofactor specificity probability.

According to another embodiment of the invention, the amino-acid sequence of the target enzyme having the cofactor specificity switched from the first cofactor to the second cofactor is determined by the steps of:

I)   before the interaction matrix is provided to the classifier, particularly the
        support vector machine in step v), determining particularly using the
        interaction matrix of the target enzyme and the impact matrix or maximum
        impact matrix, N amino-acid residues of the target enzyme with the N
        largest feature weights for the first cofactor, wherein N is a natural
        number, particularly 10,

II)   repeatedly performing a stochastic evolutionary method for replacing the
        N amino-acid residues with other amino-acid residues in the interaction
        matrix, and for each cycle of the stochastic evolutionary method and for
        each amino-acid sequence retrieved from the cycle determining the
        cofactor specificity and the cofactor specificity probability with step v) with
        the classifier,

III)   after step v), selecting at least one target enzyme with a switched
        cofactor specificity.

With the selection of the N, particularly ten most suitable mutable residue positions
for cofactor specificity reversal respectively switching, and given the possibility of
each residue position being mutated by the remaining particularly 19 amino acid
residues, it is computationally expensive to predict/determine the cofactor specificity
of every mutant combination for each theoretically possible enzyme variant.

To overcome this issue, the stochastic evolutionary method particularly implements a
stochastic evolutionary algorithm. These optimization algorithms perform an evolution
of a population by mimicking biologic events such as natural selection, here for
example the random replacement of one or more of the N amino acids.

According to another embodiment of the invention, the evolutionary algorithms used
in the stochastic evolutionary method for efficiently predict the optimal set of
mutations to reverse cofactor specificity are implemented using *inspyred* [16], an
open source framework for creating biologically-inspired computational intelligence
algorithms in Python.

According to another embodiment of the invention, the stochastic evolutionary
method executes a predefined number of evolution cycles, particularly 100 evolution
cycles.

According to another embodiment of the invention, a plurality of evolutionary
algorithms, particularly thee or five evolutionary algorithms, are comprised in the

stochastic evolutionary method, wherein a first evolutionary algorithm replaces only one of the amino acid residues of the N amino acid residues having the highest feature weight for the first cofactor during a cycle of the stochastic evolutionary method, wherein a second evolutionary algorithm replaces two of the amino acid residues of the N amino acid residues having the N highest feature weights for the first cofactor during a cycle of the stochastic evolutionary method, wherein a third evolutionary algorithm replaces three of the amino acid residues of the N amino acid residues having the N highest feature weight for the first cofactor during a cycle of the stochastic evolutionary method and so on for the rest of the remaining evolutionary algorithms of the plurality of evolutionary algorithms.

The amino acids are replaced in the interaction matrix by randomly chosen amino acid.

According to another embodiment an elitism value was set to 2, keeping the best 2 scoring enzyme variants for the next cycle, wherein the score is determined form the cofactor specificity probability.

According to another embodiment, the next best scoring 50% of all enzyme variants, particularly 50 enzyme variants, are recombined using mutation operators, with a crossover rate of 0.9 and a mutation rate of 0.1. The crossover operator particularly uses the parameters of two variant enzymes and combines them, generating two new enzyme variants, while the mutation operator substitutes one element of the enzyme variant by another, randomly generated, wherein the remaining lowest scoring 48% of enzyme variants are discarded and newly generated enzyme variants with random mutations in the available mutable positions are incorporated in the population. The stochastic evolutionary method is particularly terminated when the maximum number of cycles is reached.

According to another embodiment of the invention, in step II) selected amino sequences are provided to a next cycle of the stochastic evolutionary method, wherein the amino-acid sequences are selected based on the highest cofactor probability for the second cofactor.

This allows a convergence of the stochastic evolutionary method to a switched enzyme variant.

According to another embodiment of the invention, the first cofactor is selected from one of the redox pairs NAD / NADH and NADP / NADPH, and the second cofactor is

the other of the redox pairs, or vice versa, and wherein the target enzyme or the enzyme variant is either specific to NAD(H) or NADP(H).

NADH is also referred to NAD(H), NADPH is also referred to as NADP(H) and the two cofactors are also referred to NAD(P)(H) in an indiscriminatory fashion.

5   According to another embodiment of the invention, the first cofactor is selected from one of the redox pairs FAD / FADH2 and NADP / NADPH, and the second cofactor is the other of the redox pairs, or vice versa. FAD is short form for a flavin adenine dinucleotide.

According to another embodiment of the invention, the first cofactor is selected from 10   one of the redox pairs FAD / FADH2 and NAD / NADH, and the second cofactor is the other of the redox pairs, or vice versa.

According to another embodiment of the invention, the target enzyme and/or the enzyme variant is synthesized or the amino-acid sequence of the enzyme variant and/or the determined cofactor specificity of the target enzyme or the enzyme variant 15   is stored in an electronic storage or provided to a user of the method.

The problem is furthermore solved by a computer program and/or a computer program product comprising particularly the computer program instructions which, when the program is executed by a computer, cause the computer to carry out the method according to the invention.

20   The computer can for example be general purpose computer.

In the following exemplary embodiments of the inventions are disclosed fore illustrative purposes.

<u>Figure description</u>

In Figure 1 a cofactor neighbor residue profile matrix generation is schematically 25   depicted. Starting with the target enzyme-cofactor structure, the location of the cofactor as a reference is selected and the positions of the selected cofactor atoms are registered. For each selected cofactor atom the surrounding amino acid residues are selected, particularly those within 6 Å. The complete process is performed

automatically. The end result encompasses a matrix of interactions between each cofactor atom and neighboring residues. The presented enzyme structure depicts a Dihydropteridine Reductase bound to NAD+ from Rat liver, with the EC 1.5.1.34. PDB id: 1DIR.

In the CNRPM the cofactor atoms are listed along the rows, wherein the amino acids are listed along the columns.

Figure 2 shows a flow diagram of an exemplary execution of the method according to the invention for target enzyme cofactor specificity prediction, also referred to as NiCofactor. Three input steps are performed upon provision of the amino acid sequence of the target enzyme that are related to file handling and data conversion to a suitable data format that is referred to as "Seq1.PIR" . In order to determine the estimated the target enzyme-cofactor structure, the sequence is used for a homology search for identifying a molecular structure that is close, i.e. whose enzyme has a most similar amino acid sequence, the molecular structure associated to the enzyme having the highest degree of homology is then selected and used as a molecular structure template.

In a next step, the target enzyme sequence is aligned (step "sequence alignment and Model building") with the molecular structure template in order to generate a presumable structure of the target enzyme bound to one of the cofactors.

The aligned sequence and the associated target enzyme-cofactor structure is then transferred in a PDB file format ("Seq1.PDB"). This format is particularly suitable and common for protein structures.

From the target enzyme-cofactor structure, the interaction matrix is determined by counting the amino acid residues of the target enzyme within a predefined distance, for example within 6A, of each selected co-factor atom (steps "Cofactor neighbour residues search" and "CNRPM"). The interaction matrix, particularly the entries of the interaction matrix, CNRPM, serve as features for the trained support vector machine and are therefore provided ot the SVM classifier. The support vector machine outputs a cofactor specificity probability for the target enzyme for being specific to one of the two cofactors. This is done n form of a classification score. If the score is above a predefined threshold value the target enzyme is considered to be specific for the respective cofactor.

Figure 3 shows schematically the process of identification of the amino acid residues of a target enzyme that have the highest features weights. With the CNRPM and the

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maximum impact matrix the ten amino acids having the highest feature weight are identified.

Some or all of these amino acid residues are replaced with another amino acid. Depending on the embodiment – a deterministic replacement method or a stochastic evolutionary method is applied.

In Figure 4 an embodiment of the stochastic evolutionary method is shown for replacing amino acids in the target enzyme to generate an enzyme variant with a switched cofactor specificity.

An initial population of altered enzymes is generated by randomly replacing at least one amino acid residues from the ten highest scoring amino acid residues of the target enzyme. Five evolutionary algorithms are used, wherein each evolutionary algorithms replaces a different number of amino acid residues, between one and five.

Form this initial population comprising 100 altered enzymes, the cofactor specificity is determined according to the method of the invention in an "Evaluation step".

In this evaluation step, the two highest scoring altered enzymes are selected as elite candidates, the next 50 highest scoring altered enzymes are altered again by means of recombination provides. The score is particularly the cofactor specificity probability for the other cofactor. The remaining 48 altered enzyme having the 48 lowest scoring cofactor specificity probabilities for the other cofactor are discarded and new random mutations are introduced so that again 100 altered enzymes are provided for the next cycle. This process can be executed for each of the five evolutionary algorithms, such that for each number of replaceable amino-acids the best scoring altered enzyme can be identified. The stochastic evolutionary method is executed for 100 cycles. The best altered enzymes are the altered enzyme with the highest cofactor probability for the other, i.e. second cofactor (wherein the original target enzyme is specific to the first cofactor).

In the following various examples and exemplary embodiments for the reduction to practise are given.

1. Examples of cofactor specificity determination

1.1.1 Structure analysis and CNRPM generation

The generation of the cofactor neighbor residue profile matrix (CNRPM) for each NAD(P)(H) bound enzyme structure is built using the python computer programming language. Each target enzyme-cofactor structure is automatically handled and the distances between each selected cofactor atom and the amino acid residue

neighbourhood (surrounding) are retrieved using the PDB module of the Biopython package [2].

Interactions between cofactor atom and neighbor residue are assembled in an interaction matrix and outputted, in order to be processed by the machine learning algorithm, particularly the classifier, more particularly the support vector machine.

1.1.2 CNRPM dataset extraction

All enzymes bound to one of the following ligand IDs: NAD/NAI/NAP/NDP, representatives of NAD^+/NADH/$NADP^+$/NADPH respectively, were sought after in the PDB and automatically retrieved and analyzed using the PDB module of the Biopython package.

Entries whose enzyme or cofactor structure were incomplete or disrupted were discarded. In order to overcome the problem of overfitting/biasing with structure duplicates or point mutations of the same enzymes with different entry codes, a redundancy threshold was set and applied to the sequences coding the retrieved enzyme structures. The selected threshold was set to 95% similarity in 90% of the sequence length, allowing the removal of duplicates and point mutations of the same enzymes.

1.1.3 Machine learning

Machine learning was used for solving the classification problem in the form of supervised learning. A support vector machine, the selected method, is applied using the scikit-learn library for python [3]. LIBSVM was the employed library and the radial basis function (RBF) was the chosen kernel function.

The developed CNRPM dataset was used as a training set and handled with the NumPy library for python [4]. Model performance was evaluated by measurements including accuracy, precision, Matthew's correlation coefficient (MCC) and area under curve of the receiver operating characteristics (AUC ROC). Accuracy refers to the closeness of a measured value to a standard or known value, precision refers to the closeness of two or more measurements to each other. MCC measures the prediction quality, taking into account over- and under- predictions and giving a complementary measure of the prediction performance [5]. MCC of 1 means a perfect prediction, and 0 denotes a completely random prediction. The receiver operating characteristic (ROC) curve [6], plots true positive rate on the y-axis against the false positive rate on the x-axis. The normalized area under curve of the receiver

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operating characteristics (AUC ROC) states a perfect prediction if the AUC value is 1, and a random guess if the value is 0.5.

1.1.4 Comparative modeling for structure analysis

Homology models are created using Modeller [7] and the modeller package for python, where sequence similarity search for template selection is performed using the Smith-Waterman local alignment [8], [9] in a local database composed by structures from PDB bound to one of the cofactors NAD(P)(H). Structural similarity evidencing a suitable template was assume when two proteins share a sequence identity above 25%

The structure of the Cofactor is correctly allocated in the modelled structures by allowing Modeller to transfer these molecules from the template to the modelled structure.

1.1.5 NiCofactor tool construction

The method for determining the cofactor specificity of an target enzyme is termed NiCofactor allowing high throughput NAD(P)(H) cofactor specificity prediction was built using the python programming language. For each sequence in the FASTA format used as input, the method initiates an individual project. The steps for generating CNRPMs and performing machine learning were also integrated in NiCofactor. Results are outputted by attributing to each analyzed sequence a cofactor specificity prediction and subsequent prediction score, indicative for the cofactor specificity probability.

1.1.6 NiCofactor result validation dataset

Curated information on cofactor, cofactor specificity, EC number, organism, sequence, literature and source information on enzymes using NAD(P)(H) were retrieved automatically from brenda-enzymes using SOAPpy, a tool for building SOAP clients and servers, implemented in python [10].

1.2 Results

1.2.1 Cofactor neighbor residue profile matrix (CNRPM) development

Characterizing structural information is a challenging task due to the overwhelming amount of information associated with the structure of a protein. The main focus was to retrieve all possible interactions between each cofactor atom and the nearest residues in the binding pocket of the target enzyme. With that in mind a tool that,

given a characterized structure bound with NAD(P)(H) (in the PDB format), automatically returns a matrix of interactions between each cofactor atom and the surrounding amino-acid residues, at a distance of 6 Å. By ignoring the atoms related to the phosphate in the adenosine moiety of NADP(H), it is possible to create similar cofactor neighbor residue profile matrices (CNRPM) for both NAD(H) and NADP(H) cofactors, which is crucial to a well performing machine learning method, such as the support vector machine. Figure 1 depicts the cofactor neighbor residue profile matrix building process.

In these CNRPM, where each line refers to a selected cofactor atom (44 atoms) and each column refers to one of the twenty natural amino-acids, each value refers to the number of residues found. If, within the surroundings of an atom, a specific residue is not present, the value of that interaction is set to 0 (zero) in the interaction matrix. Thus, an interaction matrix with 20x44 entries is generated that encompasses 880 interaction values.

1.2.1.1 Building a comprehensive and representative CNRPM dataset

With the intent of applying the developed method in the construction of an accurate and representative dataset of CNRPMs, for unveiling the molecular determinants of cofactor specificity, a database of enzyme structures bound to NAD(P)(H) is assembled. To do so, (in January 13th 2016) all enzyme structures bound to one of the cofactors NAD(P)(H) from the PDB were retrieved and analyzed. The total amount of structures collected was 2742, from which 148 were discarded due to incompleteness. With the removal of protein sequence redundancy, the final dataset encompassed 921 structures, being 491 structures bound to NAD(H) and 430 to NADP(H). Once the database was assembled and validated, the developed method was applied to all structures and a CNRPM was retrieved for every enzyme.

1.2.2 CNRPM dataset analysis and processing using Machine learning

Having built a large representative dataset of 921 CNRPMs, a support vector machine (SVM) algorithm was used to attribute cofactor preference/specificity based on the CNRPMs, while evaluating the performance of the method. The SVM training algorithm works by building a model, with categorized training examples, such as the CNRPMs (which are categorized as belonging to NAD(H) or NADP(H)), and representing them as points in a high-dimensional hyperplane, separated by category and divided by a clear gap between them. This allows the algorithm to assign a category to uncategorized new examples, based on the side of the hyperplane they

fall. Performance is assessed by measuring how fine the division of categories is achieved [11].

By applying this algorithm to the CNRPMs dataset as a training set, an SVM model was created whose evaluation and performance parameters can be found in table
5   1.1. The created model achieved an accuracy of 96.2%, being able to correctly classify 886 CNRPMs as corresponding to NAD(H) or NADP(H) cofactors, with a precision of 96.03% and a Matthews correlation coefficient (MCC) of 0.92. The computed area under the receiver operating characteristic curve (AUC ROC) coefficient is 0.96. The confusion matrix displayed in table 3.1 evidences the high
10   sensitivity and specificity of the model, with similar misclassification values in both NAD(H) and NADP(H) CNRPM.

Table 1.1 depicts the evaluation and performance parameters of the created SVM model. Accuracy, precision, MCC (Mathews correlation coefficient) and AUC ROC (area under the receiver operating characteristic curve) values (top) display the
15   overall performance of the mode, indicating a well performing model. The Confusion matrix (bottom) evaluates sensitivity and specificity of the model.

Table 1.1

	Accuracy	Precision	MCC	AUC ROC
SVM model	96.20%	96.03%	0.92	0.96

Real cofactor			
NAD(H)	NADP(H)		
474	17	NAD(H)	Predicted cofactor
18	412	NADP(H)	

These results put in evidence that the type and number of residues present in the
20   cofactor binding site have a crucial role in the specification of cofactor preference in the enzyme. Such results also demonstrate the possibility to predict/indicate cofactor preference in an enzyme by analyzing its cofactor neighbor residue profile using the method according to the invention.

1.2.2.1 SVM feature weights extraction and interpretation

25   The SVM model training works by attributing weights to features in the dataset (in this case a feature is a cofactor atom-residue interaction), allowing the correct separation of the instances in the hyperplane. Such separation is what enables the

algorithm to classify a CNRPM as originated from an enzyme bound to NAD(H) or NADP(H). The extraction and interpretation of such metrics are of great importance in the identification of the crucial interactions between residue and cofactor atoms, and should allow to exactly pinpoint the set of relations in the CNRPM responsible for providing the cofactor preference to an enzyme. The extracted data, composed by 880 features and their respective weight in the SVM model, are presented in table A1 of the appendix. The highest extracted weight values correspond to 0.44991 for NADP(H) and 0.23609 for NAD(H). Despite the large amount of features, feature weight values from both NADP(H) and NAD(H) decrease rapidly from the heaviest values, leveling out in lighter features. This indicates that, despite the contribution of all features to the classification of the CNRPMs, some relations have a more significant role in classifying cofactor preference than others.

When analyzing the results, it was possible to observe that selected cofactor atoms from all parts of the cofactor structure contribute to specificity, despite the only difference between both cofactors being the presence of a phosphate molecule in the ribose from the adenine moiety. In fact, the fifteen heaviest features for both cofactors encompass atoms from adenine, ribose from adenosine, phosphates, ribose from nicotinamide ribose and nicotinamide.

Table 1.2 displays the fifty heaviest features for each cofactor along with the respective weight.

Table 1.2 shows the SVM model feature weight distribution for NAD(H) (left) and NADP(H) (right). Feature weight is distributed in a decreasing order, starting from the heaviest. Columns depict the type of atom, amino acid (AA) and feature weight. Feature weights are divided into two sub columns for each cofactor.

NAD(H)						NADP(H)					
Atom	AA	Weight	Atom	AA	Weight	Atom	AA	Weight	Atom	AA	Weight
C8A	ASP	0.236	C4B	ARG	0.144	O2B	ARG	0.450	C4N	LEU	0.145
O4B	SER	0.214	N3A	ASP	0.143	O2B	SER	0.265	C4A	ASN	0.137
C4B	ASP	0.212	O2B	LEU	0.142	O2B	LYS	0.262	N9A	TYR	0.137
C5B	ASP	0.212	O3B	GLU	0.141	C2B	ARG	0.229	O2D	ALA	0.135
O5B	ASP	0.202	N9A	LEU	0.139	O3	GLY	0.223	O2A	ALA	0.134
C5A	LYS	0.199	C1B	ALA	0.137	O3B	ALA	0.218	O5B	SER	0.133
O2D	VAL	0.193	C4D	TYR	0.135	C4D	ASN	0.217	N1A	PRO	0.132
C2B	GLU	0.179	O1N	ARG	0.134	O1A	ALA	0.186	N7N	ASN	0.129
N6A	ALA	0.179	C3N	GLU	0.128	O2B	GLY	0.180	O1A	LYS	0.127

C5B	TRP	0.177	O5D	ALA	0.128	N1A	SER	0.168	N7N	ALA	0.127
O1N	LEU	0.176	N7N	ILE	0.126	C5B	LEU	0.164	O3B	LYS	0.124
C2B	GLY	0.174	N9A	GLU	0.125	C1B	ARG	0.164	C5D	ALA	0.123
N7A	PHE	0.165	O1A	ARG	0.125	O3D	TYR	0.161	C2B	SER	0.123
O2N	LEU	0.162	O3B	ILE	0.125	O7N	CYS	0.159	C4N	GLY	0.119
N7N	ASP	0.157	C8A	MET	0.122	PN	GLU	0.159	C8A	TYR	0.117
O2N	PHE	0.157	O5D	THR	0.122	C2N	ILE	0.158	O3	ASN	0.116
O3B	PHE	0.155	O1N	ASN	0.121	C5A	TYR	0.157	O4D	THR	0.115
C5B	ALA	0.153	O7N	ARG	0.120	O2N	ASP	0.153	C4A	ALA	0.114
O3B	GLY	0.152	N3A	LYS	0.120	C3N	LYS	0.150	C4B	LEU	0.114
O2B	GLU	0.152	N1A	PHE	0.120	O5D	ILE	0.149	N1N	ASP	0.114
O1N	TYR	0.152	N9A	ILE	0.120	C4D	THR	0.148	C5B	CYS	0.113
N3A	TYR	0.151	C3B	ILE	0.118	N7A	TYR	0.148	O4B	GLY	0.112
N9A	ASP	0.150	C1B	SER	0.118	C2B	LYS	0.147	C2B	THR	0.112
O5D	GLN	0.149	C5A	PRO	0.117	O1A	SER	0.146	O7N	HIS	0.111
C2B	PRO	0.148	C3D	HIS	0.117	C6A	VAL	0.145	C8A	SER	0.110

In Table 1.2, not only cofactor atoms from the entire cofactor structure are present, but also a large majority of the 20 natural amino acids residues are present in features from both cofactors. In the case of NAD(H), besides Aspartate, also Glutamate, Alanine, Leucine, Phenylalanine, Arginine and Isoleucine residues are frequently present in the displayed features, dispersed in interactions with atoms from the entire NAD(H) structure, being Cysteine the only amino acid residue not present in the first fifty features. In the case of NADP(H), again the most important interactions occur in atoms belonging to the adenosine moiety, with Arginine residues near the atom O2B being the heaviest feature, possibly due to the presence of the phosphate connected to that atom in NADP(H). Serine, Lysine, Glycine, Alanine, Asparagine and Tyrosine residues are the most frequent amino acid residues present in the first features, being absent from this group Tryptophan, Phenylalanine, Glutamine and Methionine residues.

1.2.3 NiCofactor cofactor specificity prediction method development

For prediction of target enzyme cofactor specificity for enzymes with an unknown target enzyme-cofactor structure, comparative modelling methods are used, as these methods not only allow processing newly sequenced enzymes or organisms, but also cope with the large existing gap between available sequences in Uniprot (93 million) and structures in PDB (almost 135 thousand, with only 42572 being directly linked to

Uniprot as of October, 2017). To do so, a method was developed that implements functions for comparative modelling of protein structures using Modeller [7], a software that performs modelling by satisfaction of spatial restrains, through sequence alignment of the target sequence and known related structure templates.

5   Through the integration of the developed methods with the resulting SVM model, a method was created that automatically performs cofactor preference prediction. With only the input of an amino acid sequence, a machine learning analysis of the modelled structural environment around the cofactor is performed. Figure 2 represents the pipeline developed within the built framework that enables the

10   prediction of cofactor specificity. The developed framework is implemented in a computer program.

Figure 2 shows the developed framework pipeline. The displayed planes depict the sequence of events for cofactor specificity prediction. Starting from the left top, a Fasta file composed by the target enzyme amino acid sequence is provided to the

15   method according to the invention, where they are structurally modelled, analyzed and classified.

1.2.3.1 Validation of NiCofactor cofactor specificity prediction tool using curated information

In order to validate the developed method and the machine learning model, a dataset

20   with curated information on enzyme specificity was constructed. For that, Brenda-enzymes [1] was used and curated information on cofactor, cofactor specificity, EC number, organism, sequence, literature and source were retrieved. Firstly, the database was filtered for enzyme entries with NAD^+, NADH, $NADP^+$ or NADPH as cofactor, subsequently the cofactor commentary field was filtered for expressions

25   indicating high cofactor specificity, such us, "absolute specificity", "specific", "totally specific", "dependent on", "strict", "no activity" or "required". This step enabled us to create a dataset of 404 distinct amino acid residue sequences of different enzymes with high cofactor specificity experimentally determined, originated from a combination of 198 EC numbers and 180 organisms. From the total amount of

30   enzyme amino acid sequences, 189 are specific for NAD(H) and 215 for NADP(H). With the retrieved information present on the dataset, the amino acid sequences encompassed in the dataset were arranged and displayed in Fasta format, in a single Fasta file. This file was then uploaded and processed in the developed tool NiCofactor and predictions of NAD(P)(H) cofactor specificity were performed.

When analyzing the results obtained from the developed tool it was possible to observe that from the total 404 sequences analyzed, composing the dataset of curated information, the developed tool performed a cofactor prediction for 327 (81%) as around 11% (45) of the enzymes analyzed had their structure characterized and

5   approximately 70% (282), despite not having their structure characterized, were found to have a suitable structural template, enabling structure inference by homology modeling. For 19% (77) of the enzymes analyzed, no structural template was found, impairing the possibility of a cofactor prediction. The overall accuracy in prediction of the method according to the invention was 83.5%. By further analyzing

10   the machine learning model prediction output, it is possible to retrieve the predictions probability, which is an estimate from the model on how probable the prediction is correct. When plotting the prediction probability results we observed that the accuracy of the model tends to increase with the prediction probability, which opens the possibility of establishing a probability threshold that should improve the

15   framework predictive capabilities.

The vast majority of predictions made by the SVM, have a very high probability score, with nearly 50% of the analyzed sequences having a cofactor prediction probability of at least 95%, according to the model. In fact, 73.4% (240) of the outputted predictions have a prediction probability above 80%. This results indicate

20   that most of the prediction performed by the developed SVM model have a high probability of being correct. It is also possible to observe that the accuracy of the predictions made increases with the prediction probability score outputted by the model, which validates the prediction probability score. When the outputted prediction has a probability of at least 80%, the accuracy of the predictions increases

25   to from 83.5% to 90%, and when the prediction probability surpasses 95%, model accuracy is 96%. The presented results validate the developed tool for performing NAD(P)(H) cofactor preference predictions on enzymes, using only the enzyme's amino acid residue sequence as input, which enables the prediction of cofactor preference in newly sequenced enzymes, or enzymes whose structures are yet to be

30   characterized. To improve the prediction accuracy of the developed tool, a prediction probability threshold of 80% was set, which means that if the prediction probability of an analyzed sequence is, at least, 80%, the prediction is accepted as correct.

1.2.3.2 NiCofactor sensitivity analysis with case studies

In order to demonstrate the performance sensitivity of the method two experimentally

35   characterized case studies encompassing homologue enzymes using distinct

cofactors, were further analyzed. First, the case of *Azospirillum brasiliense's* α-Ketoglutaric Semialdehyde Dehydrogenase Isozymes (KGSADH) is presented. According to Watanabe *et al.* [12] in *A. brasiliense*, KGSADH is involved in the conversion of α-ketoglutaric Semialdehyde to α-Ketoglutarate in an alternative pathway of L-arabinose metabolism. In his study it is described that this bacterium encodes for two different KGSADH isozymes, D-glucarate/D-galactarate-inducible KGSADH-II and hydroxy-L-proline-inducible KGSADH-III with significantly similar sequences. After physiological characterization, they revealed that KGSADH-II and KGSADH-III showed similar high substrate specificity for α-ketoglutaric semialdehyde and different cofactor specificity, being KGSADH-II, NAD$^+$ dependent and KGSADH-III, NADP$^+$ dependent. KGSADH-II and KGSADH-III have a sequence identity of 62.41%, with 332 identical residues in an alignment length of 532.

The second case presented regards to two alkyl alcohol dehydrogenase (ADH) genes from the long-chain alkane-degrading strain Geobacillus thermodenitrificans NG80-2 characterized by Liu, et al. [13]. Both ADH1 and ADH2 are able to oxidize a broad range of alkyl alcohols up to at least C30, as well as 1,3-propanediol and acetaldehyde, and share a sequence identity of 26%. For either enzyme, both NAD$^+$ and NADP$^+$ can be used as electron acceptor. However, NAD$^+$ is the preferred cofactor for ADH1, while NADP$^+$ is the preferred cofactor for ADH2.

With the presented information we went on to perform cofactor prediction and assess the capability of the developed tool to predict the cofactor preference of such similar enzymes. With none of the structures of the analyzed enzymes characterized, the framework applied structure modelling in order to perform a prediction. After processing all sequences using NiCofactor for performing cofactor predictions, the resulting output was analyzed. The method according to the invention classified the analyzed enzymes correctly, being KGSADH-II classified as NAD(H) binding with a prediction probability of 99.2% and KGSADH-III as NADP(H) with 64.7% probability, whereas for the ADH genes, ADH1 was classified as NAD(H) specific with a prediction probability of 80.6% and ADH2 predicted as NADP(H) specific with 95.7% of probability.

These results demonstrate the robustness of the method according to the invention, in correctly attributing NADP(P)(H) cofactor preference to enzymes, using the amino acid sequence of the target enzyme as input. The results from the performed predictions are displayed in table 1.3. In the case of KGSADH, possibly due to their similarity, the selected structure template for both enzymes was the same, 1EZ0.pdb, a NADP$^+$ dependent Aldehyde dehydrogenase from *Vibrio harveyi*, characterized by

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Ahvazi *et al.* [14]. This enzyme has a sequence similarity of 48% with KGSADH-II and 47% with KGSADH-III, being its structure characterized with NADP$^+$ in the binding pocket. Regardless of the type of bound cofactor in the template enzyme structure, the developed method was still able to correctly classify cofactor preference in the subject enzymes, being the prediction with higher probability from the opposite cofactor. ADH1 structure was modelled using an alcohol dehydrogenase structure from *Thermotoga maritima* (PDB: 1O2D), with a sequence identity of 37%, while ADH2 model template was a butanol dehydrogenase also from *Thermotoga maritma* (PDB: 1VLJ), with 48% sequence identity.

Table 3.3 - Cofactor specificity prediction. KGSADH II and KGSADH III from *Azospirillum brasiliense* cofactor specificity prediction analysis show the predicted cofactor and associated probability. ADH1 and ADH2 from *Geobacillus thermodenitrificans* NG80-2 cofactor specificity prediction analysis show the predicted cofactor and associated probability. Template information is also displayed with PDB ID and crystalized cofactor, as well as subject and template amino acid sequence alignment identity percentage.

Fasta_ID	Predicted cofactor	Probability	Selected template	Alignment identity %
KGSADH-II	NAD(H)	0.992	1EZ0.PDB (NAP)	48
KGSADH-III	NADP(H)	0.647	1EZ0.PDB (NAP)	47
ADH1	NAD(H)	0.806	1O2D.PDB (NAP)	37
ADH2	NADP(H)	0.997	1VLJ.PDB (NAP)	45

The fact that homologue enzymes are usually specific for only one of the cofactors impairs a deeper analysis of case studies with homologues that use different cofactors. Nonetheless, the studied cases still present a good indicator of the performance sensitivity achieved. These case studies also help demonstrating that enzymes within the same environment, and with very similar functions and sequences, do not necessarily use the same cofactors for catalysis, which is a common assumption when annotating enzymes using sequence homology information.

Table A1 – The presented table encompasses all extracted feature weights from the developed support vector machine.

NADP	weight	NADP	weight	NADP	weight	NADP	weight
O2B_ARG	0.449913142	O3B_MET	0.079733928	O1N_LYS	0.048374441	PN_PHE	0.020487316
O2B_SER	0.265379606	C5A_LEU	0.079072527	C5D_ARG	0.048085877	PN_VAL	0.020312879
O2B_LYS	0.261630434	O5D_HIS	0.078422943	O2N_PRO	0.048039874	C6A_ARG	0.020247682
C2B_ARG	0.228622965	N3A_GLY	0.078126706	C1B_GLY	0.047830205	C5A_ASN	0.020164465
O3_GLY	0.222471342	C2D_ILE	0.077926803	O4D_PHE	0.047278498	C2A_PHE	0.019981824
O3B_ALA	0.217947335	O1A_ILE	0.07767809	O5B_GLY	0.047202217	C3D_THR	0.019211148
C4D_ASN	0.21647244	C3N_GLY	0.077661844	C2N_TRP	0.046976252	O3_PRO	0.019170498
O1A_ALA	0.186032894	PA_VAL	0.076984447	O3_TRP	0.046775668	C1D_TYR	0.01907725
O2B_GLY	0.180156852	O4B_TYR	0.076807191	C4A_GLN	0.046168774	O4D_CYS	0.019057066
N1A_SER	0.167953722	C8A_ALA	0.076797825	O2B_TYR	0.046021503	PN_GLN	0.018273416
C5B_LEU	0.164328457	C1D_ASN	0.076774549	O5B_ILE	0.045863598	O2A_TYR	0.01808518
C1B_ARG	0.163757278	C3N_SER	0.076468785	C1B_TYR	0.045550284	N1N_ARG	0.017754525
O3D_TYR	0.161156286	O3D_ASP	0.076355444	N1A_ALA	0.045532792	N3A_HIS	0.017404048
O7N_CYS	0.15867393	O1N_TRP	0.07594696	O2A_PHE	0.04545846	O5B_MET	0.016805257
PN_GLU	0.158627537	O3B_ASN	0.07554993	C3D_MET	0.045343175	O4D_MET	0.016554396
C2N_ILE	0.157813268	N3A_ASN	0.07451086	C2A_VAL	0.044915605	C2D_TYR	0.016524546
C5A_TYR	0.157211897	N7N_LYS	0.074129083	C4B_THR	0.043327963	O2D_GLY	0.016109269
O2N_ASP	0.152875829	C4D_LYS	0.07406761	C5N_HIS	0.043243281	O3_TYR	0.015763729
C3N_LYS	0.149656203	C4B_TYR	0.073339554	N7A_ALA	0.04306512	N3A_VAL	0.01547717
O5D_ILE	0.14931893	N6A_ILE	0.073237285	C1D_LYS	0.042897475	O4D_HIS	0.015401676
C4D_THR	0.148308899	C5D_PRO	0.073147588	O5D_TRP	0.042875009	C3B_PHE	0.015107134
N7A_TYR	0.148010431	C1B_THR	0.072608212	O3D_LEU	0.042203353	C4B_SER	0.015050321
C2B_LYS	0.146967824	O2D_PRO	0.07220358	N9A_GLY	0.041931586	O5D_TYR	0.015017127
O1A_SER	0.145495323	C2N_VAL	0.0718949	N1A_ILE	0.04163029	O1A_GLU	0.01475175
C6A_VAL	0.145139302	C6N_ASN	0.071586964	PA_ASN	0.041202421	C8A_GLY	0.014641457
C4N_LEU	0.144604686	O7N_MET	0.071435417	C5A_SER	0.041119304	C3B_LYS	0.014594822
C4A_ASN	0.1366117	C5D_ASN	0.070929606	O7N_SER	0.040917906	C4D_MET	0.014577395
N9A_TYR	0.136561537	C6A_GLY	0.070840069	C2A_MET	0.040509358	PN_PRO	0.014217951
O2D_ALA	0.135126711	C4D_ILE	0.070215604	C6A_TYR	0.040119304	C2A_CYS	0.01413095
O2A_ALA	0.134424506	O3_ALA	0.069690483	C5A_GLN	0.039823784	C5D_TRP	0.014000081
O5B_SER	0.13329123	O1N_HIS	0.069401062	PA_ALA	0.039247128	O2A_VAL	0.013853835
N1A_PRO	0.131819349	O3D_TRP	0.068758458	C6N_ARG	0.03923571	O2A_THR	0.013831904
N7N_ASN	0.128466426	N9A_THR	0.068569727	C5N_MET	0.039171159	O3D_ARG	0.013697525
O1A_LYS	0.127108198	C6N_ALA	0.068517968	N7A_ASN	0.039122243	O2D_PHE	0.013391022
N7N_ALA	0.126482461	C7N_GLN	0.068492631	C5B_ILE	0.039102077	C5B_TYR	0.013353805
O3B_LYS	0.123755873	PA_TYR	0.067210455	C6A_SER	0.038986334	C1D_TRP	0.013208285
C5D_ALA	0.123037613	O2A_LEU	0.066556605	O2B_GLN	0.038486753	O5B_PHE	0.012902275
C2B_SER	0.122966829	O4D_TYR	0.06595204	C5B_GLY	0.038076815	O3B_PRO	0.012638936
C4N_GLY	0.11898915	O4B_ARG	0.065935256	O2B_ILE	0.037717059	N7N_SER	0.012619455
C8A_TYR	0.116865395	C4N_ARG	0.065840612	O1A_CYS	0.037609135	C4N_ASP	0.012426783
O3_ASN	0.115743489	C4N_SER	0.065697869	O3D_LYS	0.036670257	C4N_MET	0.012024085
O4D_THR	0.11469516	O2N_GLN	0.065514055	C5D_TYR	0.036588347	O2B_TRP	0.011954468
C4A_ALA	0.114322444	C1D_GLU	0.065489345	C2N_LYS	0.036131842	C2D_SER	0.011876911
C4B_LEU	0.113631168	C4N_HIS	0.065426674	C2B_PHE	0.035967943	N3A_PRO	0.011724681
N1N_ASP	0.113602654	O3D_HIS	0.064528665	C5D_ILE	0.035936608	C1D_ARG	0.011535813
C5B_CYS	0.113187311	C5N_SER	0.064480807	O2D_ASP	0.035202961	C5B_PHE	0.01140188
O4B_GLY	0.111937545	C3D_PRO	0.064476088	O4B_VAL	0.035068685	O2A_HIS	0.01126606
C2B_THR	0.111749487	C1D_VAL	0.064114033	N7A_ASP	0.034717747	O5B_LYS	0.011256246
O7N_HIS	0.110573667	N1A_ASP	0.064125337	C5N_THR	0.034520477	C2A_LYS	0.011124762
C8A_SER	0.110288278	O7N_TYR	0.064086413	C3N_ASN	0.034431891	O2A_ARG	0.01061547
O5B_VAL	0.109365284	PN_ASP	0.063669197	C7N_LYS	0.03412763	O3B_TRP	0.010596586
O3_ASP	0.108192747	C2D_ALA	0.063214388	C2D_ARG	0.034126311	N1N_PHE	0.00998952
C1B_LYS	0.108082034	O3_MET	0.062869632	N1N_TYR	0.033524849	C7N_ALA	0.00994264
C4D_LEU	0.107729603	O1A_TRP	0.062853765	C5N_TRP	0.033375128	O2B_ASP	0.009908968
C1D_THR	0.107220378	C4D_ASP	0.062836247	C1D_SER	0.032376306	N7N_TRP	0.009672741
N7N_GLN	0.107145492	O7N_PHE	0.06270243	C5B_MET	0.032376033	N9A_SER	0.009415228
C2A_ALA	0.105908542	C5B_THR	0.062417335	C6A_GLN	0.031523082	N1A_LEU	0.009285992
C3N_THR	0.105248594	C6N_MET	0.062197946	C2N_ARG	0.031276336	O5B_PRO	0.009048099
C6A_ASN	0.104591342	PN_TRP	0.061994385	PN_HIS	0.030665142	C3D_ARG	0.00856326
C5N_ILE	0.104010781	C2D_VAL	0.061925295	O2N_GLY	0.030550952	N1A_CYS	0.008427029
N7N_PRO	0.102646095	PN_LYS	0.061636747	O7N_PRO	0.030053576	C6A_LEU	0.008178712

C2N_PHE	0.10249854	C3B_CYS	0.061045003	O4D_TRP	0.029930128	C4N_GLN	0.008101571
N3A_ARG	0.101773524	O1A_MET	0.060950449	C3B_GLY	0.029781147	C4A_VAL	0.008086476
C2A_THR	0.101567253	C8A_ARG	0.060817792	N1A_ARG	0.029752999	O5D_GLY	0.007969447
N6A_GLN	0.101434154	PA_CYS	0.060276098	C7N_GLY	0.029649647	C3B_ASN	0.007766154
N1N_GLN	0.100911738	C6N_TRP	0.059803838	O2B_HIS	0.029521975	C2B_TRP	0.0072598
C2D_GLU	0.100833855	O1A_ASN	0.059279603	C3N_ILE	0.028964874	N6A_ASN	0.007186107
C2N_ASN	0.099646904	PA_PHE	0.058835203	O2N_TRP	0.028578168	C2A_ARG	0.006983245
C2B_CYS	0.098308531	O5D_PRO	0.057889556	O7N_ASN	0.028227782	C1D_PHE	0.006853563
C2N_THR	0.097889728	C4N_PRO	0.057582026	N6A_LYS	0.027873306	N7N_LEU	0.006283492
N6A_ASP	0.097880648	O4B_CYS	0.05742591	O2N_LYS	0.027710043	C5D_GLN	0.00605615
C3D_TYR	0.097789016	C4D_SER	0.057417109	C3D_LYS	0.027588124	C3N_PRO	0.005892243
C3D_ASN	0.096020327	N9A_PRO	0.056977578	N1A_GLN	0.027196561	C7N_VAL	0.005799317
N7N_ARG	0.095925003	O4B_LEU	0.056890567	N6A_VAL	0.027155871	O1A_GLY	0.00542776
PA_GLU	0.095654393	C3N_VAL	0.056484245	C2N_GLY	0.027000196	O4B_ASN	0.005285506
C2A_HIS	0.094314915	O7N_GLU	0.055914303	C7N_HIS	0.026998926	N1A_TRP	0.004883473
O4D_VAL	0.093174086	O5D_ARG	0.055821931	O7N_LYS	0.026956111	C4D_GLN	0.004734092
O3D_PRO	0.092015331	O3B_CYS	0.054819911	C3B_SER	0.026905237	N9A_ARG	0.004626553
O7N_VAL	0.091513198	C2B_TYR	0.054126689	O3D_ILE	0.026902842	N3A_GLN	0.003542283
C4N_CYS	0.089453556	C7N_MET	0.053802096	N1N_ALA	0.02674033	O3D_SER	0.003501124
C5N_ALA	0.088879631	C1B_ASN	0.05365572	PN_ARG	0.026713488	C3D_GLY	0.003266954
C1D_LEU	0.088099191	O3D_PHE	0.053469827	C3B_GLN	0.02669302	O5D_LYS	0.003223613
O4D_GLY	0.086767653	C3D_LEU	0.053229595	C5N_CYS	0.026574053	C5A_CYS	0.003056985
N7N_MET	0.086633406	N3A_MET	0.053096854	C4D_HIS	0.02642775	C6A_HIS	0.003020458
O2N_VAL	0.085984478	C7N_GLU	0.05280589	O2B_CYS	0.025727259	C5D_GLU	0.00274718
C4A_PRO	0.085348112	PA_LEU	0.0527438	C4B_CYS	0.025414997	O5B_ASN	0.002626459
O7N_ILE	0.084973072	O5B_ALA	0.052622602	O3_SER	0.02531283	O2N_MET	0.00250402
C6N_PHE	0.084854801	O3B_ARG	0.052168269	C4A_HIS	0.025217149	C2D_ASN	0.002268232
O2A_MET	0.084659482	O5D_LEU	0.052144021	O2D_GLN	0.025168633	O3_LYS	0.002168882
N7A_GLY	0.084155597	O3D_CYS	0.051815901	O1A_HIS	0.024616979	N1A_HIS	0.001854708
O2D_GLU	0.084149479	C4A_SER	0.051093903	C4D_PRO	0.024612369	O1A_PHE	0.001652292
C2A_ASN	0.083940647	PA_MET	0.051091304	C6N_HIS	0.024028726	N1N_THR	0.001652148
N7N_TYR	0.083794294	C3D_VAL	0.05093086	C6N_ASP	0.023679437	C2N_TYR	0.001482017
PA_ASP	0.083171344	N3A_PHE	0.050731905	N7N_PHE	0.023380668	O5B_CYS	0.001317108
C4D_CYS	0.083049838	O2D_TRP	0.050600777	O5B_GLU	0.023362986	C5B_SER	0.001232059
C1B_CYS	0.082678811	C2B_HIS	0.050513385	PN_MET	0.023060629	N9A_CYS	0.001219865
O2B_THR	0.082490082	C6N_GLN	0.050409382	O1N_GLN	0.022780128	C5B_GLN	0.001179896
C3N_LEU	0.081724665	N7A_SER	0.049681009	N6A_LEU	0.022696971	O2A_TRP	0.000815129
N1N_HIS	0.081721236	C5N_ARG	0.04940221	C2B_GLN	0.022212051	C5A_ILE	0.000680242
O3_VAL	0.080908382	C3B_LEU	0.049214786	C2N_SER	0.021588548	C5D_ASP	0.000586944
C3N_GLN	0.080883263	C1D_PRO	0.049053943	N1N_TRP	0.021172989	C6N_GLY	0.000356168
N1N_ILE	0.080273148	C4N_THR	0.0488634	C3B_ARG	0.021026055	PA_ILE	0.0000763
		O1A_TYR	0.048695887	O2D_CYS	0.0206318		

NAD	weight	NAD	weight	NAD	weight	NAD	weight
C8A_ASP	0.236094258	C4B_VAL	0.084678643	C2A_GLY	0.04585673	PA_GLN	0.024167113
O4B_SER	0.214267146	C4N_GLU	0.084495222	C8A_CYS	0.045674108	N9A_HIS	0.023710343
C4B_ASP	0.211914031	C1B_PHE	0.082788143	C3D_ALA	0.045302776	N7A_LEU	0.023256866
C5B_ASP	0.211619835	O2B_PHE	0.082187984	C3B_MET	0.045277989	C8A_ILE	0.023221567
O5B_ASP	0.201497995	O3_ILE	0.081837268	PN_CYS	0.045070493	C3B_TYR	0.023129246
C5A_LYS	0.199153626	O4D_GLU	0.081724154	C7N_TYR	0.044696847	C2B_VAL	0.02300447
O2D_VAL	0.192728146	C4A_LYS	0.081269877	C4D_ARG	0.044656503	O2B_VAL	0.022694831
C2B_GLU	0.1793113	C4D_GLY	0.080939478	C5A_TRP	0.044048925	C5N_TYR	0.022381938
N6A_ALA	0.178674412	C6A_GLU	0.080923251	N6A_TRP	0.044048925	C4N_TRP	0.022043873
C5B_TRP	0.177316428	C3N_TYR	0.080480348	C1D_ILE	0.043144806	O2N_ASN	0.021764038
O1N_LEU	0.176228984	C1D_CYS	0.080016833	O2D_HIS	0.042941727	C2A_SER	0.021614551
C2B_GLY	0.174419789	O4B_PHE	0.079708441	PA_ARG	0.042894018	N6A_GLY	0.02146111
N7A_PHE	0.16528297	O2D_SER	0.07945322	C5A_VAL	0.042803352	C3D_CYS	0.02135515
O2N_LEU	0.161914369	O1N_VAL	0.078945222	C2B_MET	0.042765752	O5D_GLU	0.021243463
N7N_ASP	0.156998091	C5D_SER	0.078576218	C3B_ASP	0.042701922	N7A_VAL	0.021039061
O2N_PHE	0.156590241	C6A_LYS	0.077817193	C4A_CYS	0.042634678	N1A_TYR	0.020921701
O3B_PHE	0.155184371	C8A_ASN	0.077790099	PN_ALA	0.042627867	O1N_THR	0.020897222
C5B_ALA	0.152835734	N3A_LEU	0.077742066	C5A_ASP	0.042546411	N6A_CYS	0.020775768
O3B_GLY	0.151913164	N6A_PRO	0.077616001	C2N_GLU	0.042497144	O3_GLU	0.020196977
O2B_GLU	0.151898457	C2N_ALA	0.077249401	O5B_GLN	0.042340288	C5N_LEU	0.02009685
O1N_TYR	0.151450549	C7N_CYS	0.076845666	C1D_ASP	0.041495819	C4B_GLN	0.019926272
N3A_TYR	0.150626291	O3D_GLY	0.076306045	N9A_TRP	0.04128563	C1B_GLN	0.019633755

N9A_ASP	0.149611431	C3D_GLN	0.075904853	C2A_TRP	0.041271331	N7N_GLY	0.019561879
O5D_GLN	0.148609502	C5D_CYS	0.075202986	N3A_TRP	0.041271331	C4B_ILE	0.019405753
C2B_PRO	0.147532603	C6N_GLU	0.074741328	C2B_ALA	0.041255388	C7N_ARG	0.019372093
C4B_ARG	0.143967247	C5A_THR	0.074527957	N1A_THR	0.041026461	O1A_ASP	0.019244841
N3A_ASP	0.14300716	C2N_ASP	0.074459978	C2N_LEU	0.040888207	O2D_LYS	0.019194013
O2B_LEU	0.141964964	O2A_GLY	0.073382866	O5D_VAL	0.040156633	O1N_PHE	0.019158342
O3B_GLU	0.141180163	O4B_HIS	0.073219477	O3B_GLN	0.039927468	C5D_THR	0.019113421
N9A_LEU	0.139362426	C6N_LYS	0.072117998	O3D_ALA	0.039896181	C8A_GLN	0.018997788
C1B_ALA	0.136457677	C1B_TRP	0.070639586	N1N_GLY	0.039653791	C3N_ARG	0.018983506
C4D_TYR	0.13469436	C5A_ALA	0.069382114	O3B_HIS	0.039458724	O4D_SER	0.018702347
O1N_ARG	0.133895846	O2D_THR	0.069185612	C3D_ILE	0.039401154	C3N_ASP	0.018453586
C3N_GLU	0.127710335	C3B_VAL	0.069108754	N6A_ARG	0.039362501	C6A_CYS	0.018355811
O5D_ALA	0.127675979	C5N_ASN	0.069004812	O1N_CYS	0.039281688	O3D_GLN	0.018256316
N7N_ILE	0.126107558	C5N_GLY	0.068747065	N6A_SER	0.03921673	O2N_GLU	0.01817712
N9A_GLU	0.125364201	C2B_ILE	0.0686374	C2D_MET	0.03895439	C1D_GLN	0.017972709
O1A_ARG	0.124914545	O2D_LEU	0.068182443	C5D_VAL	0.038878843	C3B_HIS	0.017919884
O3B_ILE	0.124643569	C6N_LEU	0.068135623	C5D_LEU	0.038872236	C4N_PHE	0.017696368
C8A_MET	0.122164263	O1N_GLU	0.068107524	C1B_MET	0.038286853	C2D_TRP	0.01744367
O5D_THR	0.122063447	C5B_ARG	0.068011606	O4D_PRO	0.038198556	C2D_CYS	0.016935276
O1N_ASN	0.121086105	C4D_TRP	0.067954707	O1N_PRO	0.03813499	O4D_LYS	0.016476418
O7N_ARG	0.119867381	O4D_ASP	0.066602311	N1N_SER	0.038079186	C4B_TRP	0.016383175
N3A_LYS	0.119833863	C7N_THR	0.0658177	C6A_PHE	0.038067227	C7N_ASP	0.016178585
N1A_PHE	0.119547183	C3N_HIS	0.065214102	C5D_GLY	0.038048449	PA_LYS	0.016159851
N9A_ILE	0.119507247	C8A_THR	0.065129865	C5B_ASN	0.038045075	C2A_PRO	0.016101081
C3B_ILE	0.117697785	O7N_LEU	0.064726578	C6A_ASP	0.037830366	C5N_VAL	0.016097103
C1B_SER	0.117485882	C4D_PHE	0.064478493	N7A_LYS	0.037771646	N3A_SER	0.015498109
C5A_PRO	0.116871565	C1D_ALA	0.064318425	O1A_THR	0.037593202	C3D_SER	0.015402666
C3D_HIS	0.116540224	C5D_LYS	0.063959291	C4A_ARG	0.037365628	N1N_MET	0.015128158
C8A_HIS	0.115036079	C4N_LYS	0.063207444	C6A_TRP	0.037202519	C5B_PRO	0.015098312
C1B_GLU	0.114836695	O3D_MET	0.062607471	O7N_TRP	0.037119054	C2D_LEU	0.014951664
O4B_PRO	0.114719939	O5B_LEU	0.061997954	O3D_VAL	0.036711513	O2A_CYS	0.014949863
C5A_GLY	0.114416309	O2A_ASN	0.061732721	C8A_LEU	0.036654066	O3D_GLU	0.014843767
C2A_ASP	0.113940715	N6A_THR	0.061577713	N1N_GLU	0.036633975	O2N_SER	0.014469964
C3N_ALA	0.113621507	C7N_ILE	0.061382838	C4A_ILE	0.036210335	O2B_MET	0.014381374
PN_ASN	0.113168012	O3_GLN	0.061367368	C4A_TRP	0.036192205	C3D_GLU	0.013753582
N6A_HIS	0.113024505	C2N_HIS	0.061266738	C2N_GLN	0.035567033	O2A_PRO	0.013548182
C5A_MET	0.112329829	C2N_PRO	0.060988083	C3B_TRP	0.034880319	N7A_GLN	0.012765498
PA_GLY	0.11165329	C6N_THR	0.060959542	O5B_HIS	0.03477746	C3D_TRP	0.012640582
O2A_GLU	0.111244424	PA_THR	0.060328547	C1D_MET	0.0347337	O1N_ALA	0.012390965
O4B_GLU	0.109619937	C6N_TYR	0.060307835	C5N_ASP	0.034172545	O4B_THR	0.012108389
C4D_ALA	0.108202492	N7N_VAL	0.060165214	O4B_ALA	0.034119062	O4D_GLN	0.012038157
O2B_ASN	0.108147068	O5D_CYS	0.059410492	C4A_GLY	0.033689269	N1N_ASN	0.011838299
O2D_ARG	0.108038157	C3N_PHE	0.059088755	O4D_ALA	0.033586427	N9A_LYS	0.011775881
C7N_ASN	0.107213425	O2N_CYS	0.059056218	O1N_GLY	0.033556184	C2D_GLY	0.011721179
O1N_ILE	0.106640874	N1A_ASN	0.058117727	C4B_GLY	0.033344892	O7N_GLN	0.011160851
C2A_TYR	0.106180162	PN_GLY	0.057992715	O3_PHE	0.033049727	O1N_MET	0.011066817
C8A_PHE	0.105426384	O5D_ASN	0.057907553	C3B_THR	0.032956614	C1B_HIS	0.010953044
N3A_THR	0.104176435	O2N_ARG	0.057788184	C3B_PRO	0.032916451	C3B_ALA	0.010883357
N1N_PRO	0.102629164	C4D_VAL	0.057773452	C5B_GLU	0.032826056	PN_TYR	0.010703707
O5D_SER	0.10245746	C2N_CYS	0.057688219	C7N_TRP	0.032662711	N9A_ALA	0.010443668
C4B_GLU	0.102381935	N9A_VAL	0.057370037	C5N_GLN	0.032605112	C4B_ASN	0.010442276
O7N_ALA	0.102269399	C1B_ILE	0.05698411	O4B_TRP	0.032570682	N9A_GLN	0.009324513
C5B_LYS	0.102210074	O2B_ALA	0.056981668	C4B_PHE	0.032141942	N7A_GLU	0.00883231
C2A_GLN	0.101828872	C4N_TYR	0.056651445	C5B_HIS	0.03236729	C5A_PHE	0.008825354
O1A_GLN	0.101685897	C3D_ASP	0.056555527	N1A_GLY	0.03232162	O3B_LEU	0.008819554
C8A_GLU	0.100650034	C6A_PRO	0.056529942	N3A_CYS	0.032317815	C6N_PRO	0.008639054
N7N_THR	0.099880328	C1D_HIS	0.056345666	C1B_PRO	0.031301457	N1N_LEU	0.008632498
O7N_THR	0.099664754	O3D_ASN	0.056314177	O2B_PRO	0.03121384	C5D_HIS	0.007959814
C4N_ALA	0.098701899	C4B_HIS	0.056175761	O4D_ASN	0.030787145	C2D_ASP	0.00768146
O3D_THR	0.097248115	C4A_ASP	0.055724142	N7A_TRP	0.030680017	O1A_LEU	0.007670774
O4B_ASP	0.096754876	C8A_TRP	0.055617695	PN_LEU	0.029719512	C5A_HIS	0.007650503
C8A_LYS	0.095315866	C5N_PRO	0.055392821	O2A_GLN	0.029703758	O3B_TYR	0.007502293
C1B_ASP	0.094624659	O3_CYS	0.055226603	O5D_MET	0.029487202	C2D_GLN	0.007488351
N6A_GLU	0.094601341	N6A_PHE	0.054991735	PA_TRP	0.029056729	C2A_LEU	0.007471721
N7N_CYS	0.094435354	C4A_GLU	0.05479858	O3_THR	0.029037937	O3_ARG	0.006893838
N3A_GLU	0.094044915	C2B_LEU	0.053824457	O2D_ILE	0.028969805	PN_THR	0.006589932

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C2N_MET	0.093635101	O5B_ARG	0.05370051	C2D_PHE	0.028951052	C6N_ILE	0.006317509
C7N_SER	0.093273592	C3N_TRP	0.053645898	C2D_THR	0.028863737	O3B_SER	0.00628109
N7A_PRO	0.092887422	C5N_LYS	0.053172349	C6A_THR	0.028566318	N1N_CYS	0.006183564
C2A_GLU	0.092512112	O4B_GLN	0.053113516	C7N_PHE	0.0284569	O2N_HIS	0.006143172
C8A_VAL	0.092039322	N1A_VAL	0.052949956	C2D_HIS	0.028138399	C4B_ALA	0.005844543
C2D_PRO	0.091974416	N7A_ILE	0.052668712	O2A_SER	0.027907848	O2A_ASP	0.005816768
C5A_ARG	0.091774884	C2B_ASN	0.052129331	C8A_PRO	0.027839496	O2N_ILE	0.00570703
O7N_GLY	0.091607528	O2A_LYS	0.052029338	C2D_LYS	0.027755749	N3A_ILE	0.005557604
C4A_PHE	0.091478957	O5B_TRP	0.051926206	C5B_VAL	0.027696387	C4D_GLU	0.005447283
N7A_HIS	0.091434955	PN_ILE	0.051876557	C4N_VAL	0.027305555	O1N_ASP	0.005178466
C3B_GLU	0.09139035	C3N_CYS	0.050868751	O2N_ALA	0.027160743	O3B_VAL	0.004899055
C4A_LEU	0.090872072	C4B_MET	0.050634	C7N_LEU	0.027019403	C4N_ASN	0.003532887
O3B_ASP	0.090720383	C6A_ILE	0.050325083	O2D_ASN	0.026645431	C3D_PHE	0.003504186
C4B_PRO	0.09067722	O1N_SER	0.049049516	O3_LEU	0.026576686	C6N_VAL	0.003094678
N7A_ARG	0.090528536	O7N_ASP	0.048624583	O5D_PHE	0.026565767	O5B_TYR	0.002555715
N7A_MET	0.090306467	N6A_MET	0.048577237	PA_HIS	0.026455381	C3N_MET	0.002362533
C1B_LEU	0.089465086	O4D_ARG	0.048423773	O5B_THR	0.026232191	C5N_GLU	0.002179866
C5A_GLU	0.088046164	O2D_TYR	0.048363667	C6A_MET	0.026165631	O3B_THR	0.001955582
N7A_THR	0.088005608	PA_PRO	0.048103131	C4B_LYS	0.026110914	O1A_PRO	0.001622565
N7N_GLU	0.087465764	C6N_CYS	0.047847513	C5D_MET	0.025805668	C1D_GLY	0.001541014
N3A_ALA	0.087417513	C6N_SER	0.047755285	N1A_LYS	0.025796286	C4A_TYR	0.001506384
N9A_MET	0.087399922	O2D_MET	0.047508299	C5N_PHE	0.025618651	O4B_ILE	0.000794275
N9A_ASN	0.08732569	O2A_ILE	0.047073498	N1A_MET	0.025546119	N7A_CYS	0.00025527
O2N_TYR	0.087107984	PA_SER	0.046824837	C4A_THR	0.02553769	O5D_ASP	0.000240182
N9A_PHE	0.086900914	N7N_HIS	0.04665957	N1A_GLU	0.025466655	O4D_LEU	0.000196388
O3_HIS	0.08674575	C1B_VAL	0.04659479	C2A_ILE	0.025021287	C5D_PHE	0.000107303
O4D_ILE	0.086687568	C7N_PRO	0.04631959	C4A_MET	0.024798013	PN_SER	0.000103847
O2N_THR	0.086066019	O4B_MET	0.046008828	C4N_ILE	0.024773603	O1A_VAL	0.0000672
C6A_ALA	0.085894582	N6A_TYR	0.045938949	O4B_LYS	0.024320924	N1N_VAL	0.0000641
C2B_ASP	0.085308204						

2.0 Examples of switching the cofactor specificity of an enzyme

Here, detailed embodiments of a method for the in silico efficient conversion of NAD(P)(H) cofactor specificity in structurally non-characterized enzymes are disclosed.

Firstly, a method for identifying and producing an ordered list containing the most influential residues for cofactor specificity in a given enzyme structure was developed, using the SVM predictive model and CNRPM (Cofactor Neighbor Residue Profile Matrix) as elaborated previously. The created list is assumed as containing the theoretical optimal set of residue positions suitable for point mutations conferring cofactor specificity reversal.

Secondly, two methods were developed in order to identify the optimal set of point mutations required for achieving cofactor specificity change. For that, two distinct approaches were implemented, being one deterministic, using the gathered information on the most influential residues for both cofactor specificities; and the

other stochastic, using evolutionary algorithms to locate the optimal set of mutations capable of reverting cofactor specificity.

2.1 Methods

2.1.1 Aminoacid residue sequences and protein structure templates

5 The wild-type amino acid residue sequences from the selected case-study enzymes were retrieved from Uniprot, while mutant sequences were replicated in silico according to the specifications presented in the literature. Uniprot IDs from the four selected enzymes are as following: Pichia stipitis's xylose reductase: P31867; Gluconobacter oxydans's xylitol dehydrogenase: Q8GR61; Pichia stipitis's xylitol

10 dehydrogenase: P22144; Tramitichromis intermedius's leucine dehydrogenase: Q60030.

Gluconobacter oxydans' xylitol dehydrogenase had its structure already experimentally characterized (PDB id: 1ZEM), being therefore used as a template for modeling the structure of the predicted mutants. As for the remaining three enzymes,

15 since their structures were not experimentally characterized, the structures of the wild-type and corresponding mutants were generated using comparative modeling. Pichia stipitis' xylose reductase structure was modeled using the homologue structure of Arabidopsis thaliana's aldo-keto reductase (PDB id: 3H7R) with 47% identity; Pichia stipitis' xylitol dehydrogenase structure was modeled using the

20 homologue structure of Homo sapiens' sorbitol dehydrogenase (PDB id: 1PL6) with 44% identity and Tramitichromis intermedius' leucine dehydrogenase structure was modeled using the homologue structure of Rhodococcus sp. M4's phenylalanine dehydrogenase (PDB id: 1BW9) with 37% identity.

2.1.2 Cofactor specificity prediction

25 Predictions on cofactor specificity change were performed using NiCofactor, the method described previously in the description, for allowing the high throughput NAD(P)(H) cofactor specificity prediction. NiCofactor was built using the python programming language. Input sequences are required to be in FASTA format. For each sequence, the tool initiates an individual project. The tools for generating

30 CNRPMs and performing machine learning were also integrated in NiCofactor.

Results are outputted by attributing to each analyzed sequence a cofactor prediction and subsequent prediction score. The default probability score threshold used is 0.8.

2.1.3 Evolutionary algorithm implementation

5   The evolutionary algorithms used in the stochastic method for efficiently predict the optimal set of mutations to reverse cofactor specificity were implemented using inspyred [16], an open source framework for creating biologically-inspired computational intelligence algorithms in Python.

Five evolutionary algorithms were implemented, with the only difference between them being the maximum candidate size allowed. Each algorithm was configured to
10   run through 100 generations, being the initial population composed by 100 individuals. Each individual was randomly created according to the candidate maximum size, which varied between 1 and 5. This corresponds to the creation of mutant amino acid residue sequences derived from the original target aminoacid residue sequence, containing between 1 and 5 mutations each. Elitism value was set
15   to 2, keeping the best 2 scoring individuals for the next generation. The next best scoring 50 individuals were recombined using mutation operators, with a crossover rate of 0.9 and a mutation rate of 0.1. The crossover operator uses the parameters of two individuals and combines them, generating two new individuals, while the mutation operator substitutes one element of the individual by another, randomly
20   generated. The remaining lowest scoring 48 individuals were discarded and newly generated individuals with random mutations in the available mutable positions were incorporated in the population. The optimization process is terminated when the maximum number of generations is achieved.

2.1.4 Protein structure visualization

25   Wild-type and mutant enzyme structures were visualized using PyMol [17] a free and user-friendly molecular graphics system for molecular visualization written in Python programming language.

2.2 Results and discussion

2.2.1 Identification of mutable residue positions for cofactor specificity reversal

As previously stated, the SVM model, trained with a large dataset of CNRPMs, performed the attribution of importance scores to the features in the dataset, allowing the correct separation of the instances in the hyperplane. In this case, the features are composed of relations between the atoms in each cofactor and the corresponding neighbor amino acid residues. Then, the score, i.e. the feature weight, attributed to each feature, reveals the impact of each interaction in the binding preference of the cofactors, with higher scoring features having a higher impact on cofactor specificity. Figure 3 depicts a representation of the process undertaken for the selection of mutable residue positions for cofactor specificity reversal. Through the analysis of the CNRPM generated from a target enzyme's structure, and by combining this information with the data stored in the SVM model, the sorting of the best features for cofactor specificity is performed. When the most influential features for cofactor specificity in a given protein structure are sorted, the corresponding residue position in the sequence is retrieved and stored for each feature. When features from ten distinct residues in the sequence are retrieved, the list is closed.

The end result is an ordered list of the most influential residues in cofactor specificity for a given target enzyme, being assumed that, due to their influence in cofactor specificity, this list contains the optimal mutable residues for cofactor specificity reversal.

Despite the developed method's utility in precisely pinpointing suitable target residues for cofactor specificity reversal mutations, the computational costs or comparably high due to the combinatory amount of total mutant possibilities. In order to optimize this task, two distinct approaches were undertaken, resulting in the development of two different methods for determining the optimal set of mutations necessary to achieve cofactor specificity reversal.

2.2.2 Cofactor specificity reversal – Deterministic method

With the intent of surpassing the overwhelming amount of combinatorial mutations necessary for the screening of all suitable mutable residue positions to achieve the optimal cofactor specificity reversal mutant, a deterministic method was developed.

This deterministic approach is based on the formulation of a hypothesis regarding both cofactor's most influential features. With this in mind, the suggested hypothesis states that: if, for each atom composing a cofactor, there is a specific neighbor amino acid residue with the highest impact in the cofactor specificity, then, if this interacting amino acid residue is replaced by the amino acid residue with the highest impact for the opposite cofactor, the binding specificity should be affected. Therefore, if enough amino acid residues with high impact on cofactor specificity are changed, the cofactor specificity should be reverted.

In order to implement the stated hypothesis, the extracted SVM feature weights present in appendix, in table A1, were further examined. For each selected cofactor atom, the strongest feature present for NAD(H) and NADP(H) specificity was selected, and the associated amino acid residue retrieved. The resulting chart, displayed in table 2.1, represents, for each selected cofactor atom, the amino acid residue interaction with highest impact on cofactor specificity, and consequently the candidate for performing point-mutations in that area of the binding spot.

Table 2.1. Point-mutation selecting chart. Each selected cofactor atom and corresponding molecular localization is represented in the column "cofactor atom". For each atom, the corresponding NAD(H) and NADP(H) specificity is represented in columns "NAD(H) specific" and "NADP(H) specific" respectively. This table corresponds to the impact matrix.

Cofactor atom	NAD(H) specific	NADP(H) specific	Cofactor atom	NAD(H) specific	NADP(H) specific
PA	GLY	GLU	O3	HIS	GLY
O1A	ARG	ALA	PN	ASN	GLU
O2A	GLU	ALA	O1N	LEU	TRP
O5B	ASP	SER	O2N	LEU	ASP
C5B	ASP	LEU	O5D	GLN	ILE
C4B	ASP	LEU	C5D	SER	ALA
O4B	SER	GLY	C4D	TYR	ASN
C3B	ILE	CYS	O4D	ILE	THR
O3B	PHE	ALA	C3D	HIS	TYR
C2B	GLU	ARG	O3D	THR	TYR
O2B	GLU	ARG	C2D	PRO	GLU
C1B	ALA	ARG	O2D	VAL	ALA
N9A	ASP	TYR	C1D	CYS	THR
C8A	ASP	TYR	N1N	PRO	ASP
N7A	PHE	TYR	C2N	MET	ILE

C5A	LYS	TYR	C3N	GLU	LYS
C6A	ALA	VAL	C7N	ASN	GLN
N6A	ALA	GLN	O7N	ARG	CYS
N1A	PHE	SER	N7N	ASP	ASN
C2A	ASP	ALA	C4N	ALA	LEU
N3A	TYR	ARG	C5N	ASN	ILE
C4A	PHE	ASN	C6N	GLU	PHE

2.2.2.1 Implementation of the deterministic method for cofactor specificity conversion

The implementation of the deterministic method for the conversion of NAD(P)(H) cofactor specificity, starts with the structural analysis of the subject enzyme and respectively CNRPM assembly with NiCofactor. If the subject enzyme's structure is not characterized, homology modelling with a suitable structural template is performed automatically by NiCofactor.

Once created, CNRPM features are sorted and the one with the highest impact is selected. The amino acid residue sequence position from the residue present in the feature is retrieved, while the atom present in the feature is searched in the point-mutation selecting chart displayed in table 2.1, being the candidate amino acid residue mutant selected.

The wild-type amino acid residue is replaced by the mutant candidate in the amino acid residue sequence. The mutant sequence is retrieved and its cofactor specificity is predicted using NiCofactor.

If a prediction is performed, with a probability score above the threshold value, and the cofactor predicted for the mutant sequence is changed, the mutant sequence is accepted as having its cofactor specificity successfully altered. If, on the other hand, the cofactor prediction did not change, the conversion method continues with the mutant sequence and the second highest impact feature is used. This step is performed iteratively, with mutations being incremented in the sequence until the cofactor prediction is changed. If after 10 consecutive mutations the cofactor prediction remains unaltered, the mutation is regarded as unviable.

For example, for the NAD(H) dependent target enzyme structure, the atom-amino acid residue interaction with highest impact on cofactor specificity is originated by the presence of a Phenylalanine (F) near the atom O3B, in the ribose from the adenine

moiety. By consulting table 2.1 it is possible to observe that the residue originating the atom-amino acid residue interaction, with atom O3B, with the highest impact on cofactor specificity for NADP(H) is an Alanine (A). Being the sequence position of the selected Phenylalanine, position 42, a point mutation is performed and the Phenylalanine is substituted by an Alanine. Despite this mutation, the cofactor specificity prediction of the target enzyme was not altered, being therefore selected the second highest impact interaction, the Aspartate (D) near the atom O5B. With the substitution of Aspartate by a Serine (S) not rendering an altered cofactor specificity prediction, the third highest impact interaction was selected. This time, the Aspartate near atom C8A was mutated into a Tyrosine (Y) and the resulting mutant F42A/D23S/D71Y was successfully predicted as having reverted its original cofactor specificity.

The presented deterministic method is a fast and precise approach for the complex problem of selecting the optimal set of mutations capable of reverting cofactor specificity in a target enzyme. Despite its overall efficacy, robustness and time efficiency, the deterministic characteristics of this approach mean that there are multiple mutation combinations that are not taken into consideration, with the possibility of better results for a set of cofactor specificity reverting mutations being overlooked. Due to these constraints, and in order to analyze the highest number of mutation combinations possible, a stochastic method was developed, with the incorporation of an evolutionary algorithm.

2.2.3 Cofactor specificity reversal – Stochastic method

With the selection of the ten most suitable mutable residue positions for cofactor specificity reversal, and given the possibility of each residue position being mutated by the remaining 19 amin oacids residues, it becomes clear the impossibility of predicting the cofactor specificity of every mutant combination. To overcome this issue, a stochastic method was developed through the implementation of an evolutionary algorithm. These optimization algorithms perform the evolution of a population by mimicking biologic events such as natural selection. Each individual in a population is evaluated through a fitness function and compared with newly generated individuals created by the application of reproduction operators to selected parents. As in nature, only the fittest individuals are allowed to continue in the population and reproduce [18].

Figure 4 is depicts a representation of the implemented evolutionary algorithms. Given the target amino acid residue sequence and the list of 10 mutable positions, an initial population of 100 mutant amino acid residue sequences (individuals) was generated, with random mutations in the available mutable positions. In this work, 5 evolutionary algorithms were implemented, with the only difference being the maximum candidate size allowed, varying between 1 and 5 mutations per individual. The optimization process was run for 100 generations. During each generation, the cofactor specificity of each individual was predicted using NiCofactor. After evaluating the entire population, the two best scoring individuals were maintained for the next generation, while the next best 50 undertook a recombination process, being 90% by crossover, where two individuals are crossed over to generate two new individuals, and 10% by mutation, where an individual's suitable aminoacid residue is randomly mutated. The remaining lowest scoring 48 individuals were discarded and newly generated individuals with random mutations in the available mutable positions were incorporated in the population. In the end of the optimization process, the five mutant sequences, containing 1 to 5 mutations, with the highest cofactor prediction score for the opposite cofactor were retrieved and outputted as result.

2.2.4 Case studies

With the intent of assessing the performance of the developed methods for cofactor specificity reversal, four case studies were replicated in silico and their cofactor specificity reverted, using the developed methods. From the group of cofactor engineering studies published by Khoury and coworkers [19], the four enzymes that were found to have completely reverted specificity or largely decreased affinity for one of the cofactors, increasing the affinity of the other, were selected as case studies. These were the cases of xylose reductase from Picchia stipitis (PsXR) [20], xylitol dehydrogenase from Gluconobacter oxydans (GoXD) [21], xylitol dehydrogenase from Pichia stipitis (PsXD) [22] and leucine dehydrogenase from Tramitichromis intermedius (TiLD) [23]. For these enzymes, NiCofactor was able to correctly predict the cofactor specificity of both wild-type and specificity reversed mutants. With that in mind, the four enzymes' amino acid residue sequence were retrieved and processed using the above described methods with the intent of showcasing the results achieved in silico, and comparing them to the experimentally determined data on cofactor specificity reversing mutations.

From the four enzymes analyzed, three are part of the xylose metabolism, an extremely important pathway due to its great economical potential. Being a major component of hemicellulose and only second to glucose as the most abundant sugar in nature, D-xylose can be bioconverted from agricultural biomass wastes into biofuels, such as ethanol, through fermentation processes. However, Saccharomyces cerevisiae, the best adapted microorganism for producing ethanol, is not genetically equipped for metabolizing xylose. To solve this problem, xylose fermenting genes have been cloned in S. cereviseae from other organisms capable of metabolizing this sugar, such as Pichia spitipis [20], [22] and Gluconobacter oxidans [21]. Xylose reductase (EC 1.1.1.21) reduces xylose into xylitol using NADPH and xylitol dehydrogenase (EC 1.1.1.9) oxidizes, posteriorly, xylitol into xylulose, using NAD+. Nonetheless, this difference in cofactor specificity creates an intercellular redox unbalance, hindering ethanol production yields and promoting xylitol excretion. An elegant solution implemented to solve this problem is the cofactor specificity reversal of xylose reductase from NADPH to NADH [20] or, by alternative, the specificity reversal of xylitol dehydrogenase from NAD+ to NADP+, taking advantage of the often higher availability of NADP+ in the cell [21], [22].

The remaining enzyme, Leucine dehydrogenase from Thermoactinomyces intermedius uses NAD+ for catalyzing the reversible deamination of L-leucine to its 2-oxo analogue, 4-methyl-2-oxopentanoate. As biosynthesis reactions generally use NADP+ as cofactor, leucine dehydrogenase cofactor specificity reversal might improve this reaction's efficiency [23].

2.2.4.1 PsXR – Pichia stipitis xylose reductase

Xylose reductase (PsXR), from Pichia stipitis, was the only enzyme in the analyzed group with cofactor specificity for NADP(H), with the remaining enzymes being specific for NAD(H). In table 5.2 the results achieved for the in silico cofactor specificity change of PsXR are displayed.

As previously stated, NiCofactor was able to correctly predict the cofactor specificity from both wild-type and literature cofactor reversed mutant, being the results achieved by the deterministic and stochastic methods only outputted when the prediction score threshold is achieved.

Table 2.2. Mutations, cofactor predictions and prediction scores from literature experimental data, as well as from the implementation of both methods for reversing cofactor specificity in silico of PsXR. The deterministic method outputs only one mutant, while the stochastic method outputs five different mutants with the best found set of mutations for specificity reversal, according to the maximum candidate size allowed by the method, with the number on the gene name corresponding to the number of mutations selected.

Gene name	Mutation	Predicted cofactor	Prediction score
PsXR	Wild-type	NADP	0.8764
PsXR Literature	K270S/S271G/N272P/R276F	NAD	0.8792
PsXR Deterministic	S271E/R276E	NAD	0.9747
PsXR Stochastic 1	R276D	NAD	0.8661
PsXR Stochastic 2	K270D/S271D	NAD	0.9773
PsXR Stochastic 3	K270D/S271D/R276D	NAD	0.9996
PsXR Stochastic 4	S215R/K270D/S271D/R276D	NAD	0.9995
PsXR Stochastic 5	G217D/I268R / K270D/S271D/R276D	NAD	0.9999

When analyzing table 2.2 it is also possible to observe the amount and type of point mutations recommended by the developed methods in order to achieve cofactor specificity reversal. Being the literature mutant composed by four point-mutations, achieved through the implementation of a combinatorial active-site saturation mutagenesis method, we can observe that both deterministic and stochastic methods here implemented were able to predict mutants with fewer point-mutations and higher predicted reversed cofactor specificity. In this case, PsXR Deterministic is composed by only two point-mutations, with an Arginine (R) and a Serine (S) being substituted by a Glutamate (E). In the case of PsXR stochastic, the evolutionary algorithm was able to find a mutant with predicted reversed cofactor specificity with only one point-mutation, being this individual, due to its lower amount of point-mutations, considered the best hypothesis for performing in vivo cofactor specificity reversal. When analyzing the remaining stochastic mutants, it is observed that the mutants with higher amounts of point-mutations tend to incorporate the point-mutations predicted for the stochastic mutants with fewer point-mutations, indicating a strong effect of these mutations for the reversal of cofactor specificity. We can also see, in the stochastic mutants, that the cofactor prediction scores increase with the amount of point-mutations predicted, however, preference should be given to mutants with fewer mutations in order to preserve the structural stability of the enzyme.

2.2.4.2 GoXD –Gluconobacter oxydans xylitol dehydrogenase

Xylitol dehydrogenase (GoXD), from Gluconobacter oxydans, oxidizes xylitol into xylulose and has been shown to use exclusively NAD+ as a reaction cofactor [21]. Table 5.3 displays the results achieved for the in silico cofactor specificity change of GoXD from NAD+ dependent to NADP+.

Table 2.3. lists mutations, cofactor predictions and prediction scores from literature experimental data, as well as from the implementation of both methods for reversing cofactor specificity in silico of GoXD. The deterministic method outputs only one mutant, while the stochastic method outputs five different mutants with the best found set of mutations for specificity reversal, according to the maximum candidate size allowed by the method, with the number on the gene name corresponding to the number of mutations selected.

Gene name	Mutation	Predicted cofactor	Prediction score
GoXD	Wild-type	NAD	0.9678
GoXD Literature	D38S/M39R	NADP	0.9541
GoXD Deterministic	D38Y/A92Q/G93R	NADP	0.8822
GoXD Stochastic 1	D38R	NADP	0.8668
GoXD Stochastic 2	D38R/A92R	NADP	0.9752
GoXD Stochastic 3	D38R/D64R/A92R	NADP	0.9944
GoXD Stochastic 4	G16K/D38R/D64S/G93R	NADP	0.9957
GoXD Stochastic 5	G14S/G16T/D38R/D64R/A92R	NADP	0.9999

The results displayed in table 2.3 show a high score for the prediction of cofactor specificity of wild-type and literature experimental data, increasing the confidence level on the agreement between predicted and experimental results. In the analyzed case-study, the literature mutant was achieved with only two point-mutations, while the deterministic method required three mutations to achieve the same cofactor specificity prediction. As to GoXD stochastic results, the developed method was able to output a predicted reversed cofactor specificity mutant encompassing only one point-mutation, being it considered the best hypothesis for performing in vivo cofactor specificity reversal with minimal interventions. When further analyzing the achieved results, it is possible to observe that both the literature mutant and the selected stochastic mutant share similar features despite being originated from different approaches. From these approaches, the one described in the literature is the most laborious, involving structure characterization and structural alignment with other

enzymes, together with the multiple selections of conserved amino acid residue positions. In the literature mutant an Aspartate residue in position 38 was deleted and an Arginine residue was incorporated in position 39, whereas in the stochastic mutant, this event occurred in the same spot, position 38. A common characteristic that the predicted mutations appear to possess, in order to successfully reverting cofactor specificity in this case, is the promotion of Arginine (R) inclusion and the exclusion of Aspartate (D) in the analyzed sequences.

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Claims

1. A method for determining a cofactor specificity of a target enzyme, wherein the
    target enzyme is expected to use one of a first cofactor and a second cofactor
    based on an amino-acid sequence of the target enzyme, and/or for determining
    an amino-acid sequence of a target enzyme variant, wherein the variant is
    characterized by a cofactor specificity differing from that of the target enzyme,
    the method comprising at least the steps of:

    i)   providing an atomic structure for each of both cofactors, wherein each
          atomic structure comprises cofactor atoms, and wherein cofactor atoms
          in the atomic structures that are located at the same corresponding
          locations in both atomic structures are selected,

    ii)   providing the amino-acid sequence of the target enzyme,

    iii) determining an estimated target enzyme-cofactor structure comprising
          information on a spatial structure of the target enzyme bound to one of
          the cofactors,

    iv)   generating an interaction matrix for the target enzyme-cofactor structure,
          wherein the interaction matrix comprises entries relating the selected
          cofactor atoms to surrounding amino-acid residues of the target enzyme,
          particularly wherein for each selected cofactor atom, entries are
          generated in the interaction matrix that comprise the counts of each
          amino-acid residue of the target enzyme within a predefined distance to
          the selected cofactor atom,

    v)   determining a cofactor specificity of the target enzyme by providing the
          interaction matrix, particularly the entries of the interaction matrix, to a
          trained classifier that is configured to classify the cofactor specificity of
          the target enzyme based on the provided interaction matrix to either the
          first or the second cofactor.

2. The method according to claim 1, wherein particularly if the target enzyme-
    cofactor structure is unknown, the following steps are executed for determining
    the estimated target enzyme-cofactor structure:

    –   performing a homology search with the amino-acid sequence of the
          target enzyme in a protein structure database comprising information on
          molecular structures of enzymes bound to the first or the second cofactor,

    −   assigning the molecular structure comprising the enzyme that exhibits a highest degree of homology to the amino-acid sequence of the target enzyme as a molecular structure template,

    −   from the molecular structure template determining the target enzyme-cofactor structure for the amino-acid sequence of the target enzyme bound to the cofactor particularly by aligning the amino-acid sequence of the target enzyme to the molecular structure template.

3.   The method according to any one of the preceding claims, wherein the classifier is trained by

    −   providing information on molecular structures of a plurality of enzymes, wherein each molecular structure represents an enzyme bound to the first or the second cofactor,

    −   for each molecular structure, generating the interaction matrix, wherein each interaction matrix is associated to the respective cofactor of the molecular structure,

    −   training the classifier with the interaction matrices so that the classifier is configured to classify the cofactor specificity for the first or the second cofactor of an enzyme based on the entries of the interaction matrix.

4.   The method according to any one of the preceding claims, wherein the trained classifier provides a cofactor specificity probability for the first and/or the second cofactor for the interaction matrix for classifying the cofactor specificity of the target enzyme, wherein if said probability exceeds a predefined threshold value, the target enzyme is classified to be specific to the respective cofactor.

5.   The method according to any one of the preceding claims, wherein the amino-acid sequence of the target enzyme having the cofactor specificity switched from the first cofactor to the second cofactor is determined by the steps of:

    a)   particularly providing the target cofactor enzyme structure with a target enzyme being specific to the first cofactor,

    b)   prior to step v), replacing at least one amino-acid in the interaction matrix,

    c)   determining whether the cofactor specificity determined in step v) is switched from the first cofactor to the second cofactor,

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d)   particularly repeating the steps b) to c), particularly until the cofactor specificity determined in step v) is switched from the first cofactor to the second cofactor.

6.   The method according to any one of the preceding claims, wherein for each of the two cofactors and for each of the selected cofactor atoms and for each amino-acid, a feature weight indicative of a cofactor specificity strength is determined or provided, particularly wherein the trained classifier determines and/or provides the feature weights after being trained, particularly wherein said feature weights are provided in form of a computer readable look-up table.

7.   The method according to claim 6, wherein a maximum impact matrix is determined or provided relating each selected cofactor atom to the amino-acid having the largest feature weight for the cofactor specificity for the first cofactor and the second cofactor, particularly wherein the maximum impact matrix is determined and/or provided by the trained classifier, particularly wherein the maximum impact matrix stored as a computer readable look-up table.

8.   The method according to any one of the preceding claims, wherein the amino-acid sequence of the target enzyme having the cofactor specificity switched from the first cofactor to the second cofactor is determined by the steps of:
-    before the interaction matrix is provided to the trained classifier in step v), replacing M amino-acid residues in the interaction matrix corresponding to the amino-acid residues of the target enzyme with the M largest feature weights for the first cofactor, with the corresponding M amino-acid residues for the same cofactor atoms having the largest feature weight for the second cofactor, wherein M is a natural number,
-    particularly providing the such altered interaction matrix to the trained classifier as the interaction matrix in step v),
-    after step v), determining whether the specificity of the enzyme variant associated to the interaction matrix is switched with respect to the target enzyme.

9.   The method according to claim 8, wherein starting from an initial value for M, particularly M =1, the steps of claim 8 are repeated, wherein M is incremented

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by one or more during each repetition, until the cofactor specificity of the target
enzyme is switched or a predefined maximum value for M is reached.

10. The method according to any one of the claims 5 to 9, wherein the amino-acid
sequence of the target enzyme having the cofactor specificity switched from the
first cofactor to the second cofactor is determined by the steps of:
I)   before the interaction matrix is provided to the trained classifier in step v),
      determining N amino-acid residues of the target enzyme with the N
      largest feature weights for the first cofactor, wherein N is a natural
      number, particularly 10,
II)   repeatedly performing a stochastic evolutionary method for replacing the
      N amino-acid residues with other amino-acid residues in the interaction
      matrix, and for each cycle of the stochastic evolutionary method and for
      each amino-acid sequence retrieved from the cycle determining the
      cofactor specificity and the cofactor specificity probability with step v) with
      the trained classifier,
III) after step v) of claim 1, selecting at least one target enzyme with a
      switched cofactor specificity.

11. The method according to claim 10, wherein in step II) selected amino
sequences are submitted to a next cycle of the stochastic evolutionary method,
wherein the amino-acid sequences are selected based on the highest cofactor
probability for the second cofactor.

12. The method according to one of the preceding claims, wherein the first cofactor
is selected from one of the redox pairs NAD / NADH and NADP / NADPH, and
the second cofactor is the other of the redox pairs.

13. Method according to one of the preceding claims, wherein the target enzyme is
synthesized.

14. A computer program comprising instructions which, when the program is
executed by a computer, cause the computer to carry out the method according
to any of the claims 1 to 12.

*****

Fig. 1

	ALA	ARG	ASN	ASP	CYS	GLN	GLU	GLY	HIS	ILE	LEU	LYS	MET	PHE	PRO	SER	THR	TRP	TYR	VAL
Atom PA	2	0	0	0	0	0	0	0	5	0	0	1	0	0	0	0	0	0	0	1
Atom O1A	2	0	0	0	0	1	0	0	3	0	0	1	0	0	0	0	0	1	0	1
Atom O2A	1	1	0	0	0	0	0	0	2	0	0	1	0	0	0	0	1	0	0	0
Atom O5B	2	1	0	1	0	0	0	0	5	0	0	1	0	0	0	0	0	0	0	1
Atom C5B	2	1	0	1	0	0	0	0	5	0	0	1	0	0	0	0	0	0	0	1
Atom C4B	2	1	0	1	0	0	0	0	5	0	0	0	0	0	0	0	0	0	1	1
Atom O4B	1	1	0	1	0	1	0	0	4	0	0	0	0	0	0	0	1	0	1	2
Atom C3B	2	1	0	1	0	1	0	0	3	0	0	0	0	0	0	0	0	0	0	2
Atom O3B	1	1	0	1	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	1
Atom C2B	1	0	0	1	0	1	0	0	3	0	0	0	0	0	0	0	0	0	0	1
Atom O2B	0	1	0	1	0	1	0	0	2	0	0	0	0	0	0	0	0	0	0	2
Atom C1B	1	1	0	1	0	1	0	0	3	1	0	0	0	0	0	0	1	0	0	2
Atom N9A	1	0	0	1	0	1	0	0	2	0	0	0	0	0	0	0	1	0	0	1
Atom C8A	1	0	0	1	0	1	0	0	2	0	0	1	0	0	0	0	1	0	0	1
Atom N7A	1	0	0	0	0	2	0	0	1	0	0	1	0	0	0	1	1	0	0	1
Atom C5A	1	0	0	1	0	2	0	0	1	0	0	1	0	0	0	0	1	0	1	2
Atom C6A	1	0	0	0	0	2	0	0	1	0	0	1	0	0	0	0	1	0	1	2
Atom N6A	0	0	0	0	0	2	0	0	0	0	0	1	1	0	0	0	1	1	1	2
Atom N1A	1	0	0	1	0	2	0	0	1	0	0	1	0	0	0	0	1	0	1	2
Atom C2A	1	0	0	1	0	1	0	0	1	1	0	0	0	0	0	0	1	0	1	2
Atom N3A	1	0	0	1	0	2	0	0	3	1	0	0	0	0	0	0	1	0	1	2
Atom C4A	1	0	0	1	0	2	0	0	2	1	0	1	0	0	0	0	1	0	1	2
Atom O3	2	0	0	0	0	0	0	0	4	0	2	0	0	0	0	0	0	0	0	1
Atom PN	1	0	0	0	0	0	0	0	3	0	3	0	0	0	0	0	1	0	0	1
Atom O1N	1	0	1	0	0	0	0	0	1	0	3	0	0	0	0	0	1	0	0	0
Atom O2N	1	0	0	0	0	0	0	0	2	0	3	0	0	0	0	0	1	0	0	1
Atom O5D	1	0	0	0	0	0	0	0	2	0	2	0	0	0	0	0	0	0	0	1
Atom C5D	2	0	0	0	0	0	0	0	3	0	2	1	0	0	0	0	0	0	0	1
Atom C4D	2	0	0	0	1	0	0	0	3	0	2	1	0	0	1	0	0	0	0	1
Atom O4D	2	0	0	0	0	0	0	0	2	0	2	1	0	0	1	0	0	0	1	1
Atom C3D	2	0	0	0	0	0	0	0	3	0	1	1	0	0	0	0	0	0	0	1
Atom O3D	2	0	0	0	1	0	0	0	3	0	0	1	0	0	0	1	0	0	0	1
Atom C2D	2	0	0	0	0	0	0	0	3	0	1	1	0	0	0	0	0	0	1	1
Atom O2D	2	0	0	0	0	0	0	0	3	0	0	1	0	0	0	1	0	1	1	0
Atom C1D	3	0	0	0	0	0	0	0	3	0	1	1	0	0	1	0	0	0	1	0
Atom N1N	1	0	0	0	0	0	0	0	2	0	1	1	0	0	1	0	0	0	1	1
Atom C2N	0	0	1	0	0	0	0	0	1	0	1	1	0	0	1	0	1	0	1	0
Atom C3N	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	1	1
Atom C7N	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	2	0	1	1
Atom O7N	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	2	0	0	0
Atom N7N	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	2	0	0	1
Atom C4N	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	1	1
Atom C5N	1	0	0	0	0	0	0	0	1	0	1	1	0	0	1	0	1	0	1	1
Atom C6N	1	0	0	0	0	0	0	0	1	0	1	2	0	0	1	0	1	0	1	1

Fig. 2

Fig. 3

CNRPM

Maximum impact matrix

Ten amino acid residues having the highest feature weight

Aminoacid residue	Sequence position
Arginine	276
Serine	271
Lysine	270
Glycine	217
Alanine	229
Tyrosine	48
Isoleucine	268
Lysine	77
Serine	215
Serine	214

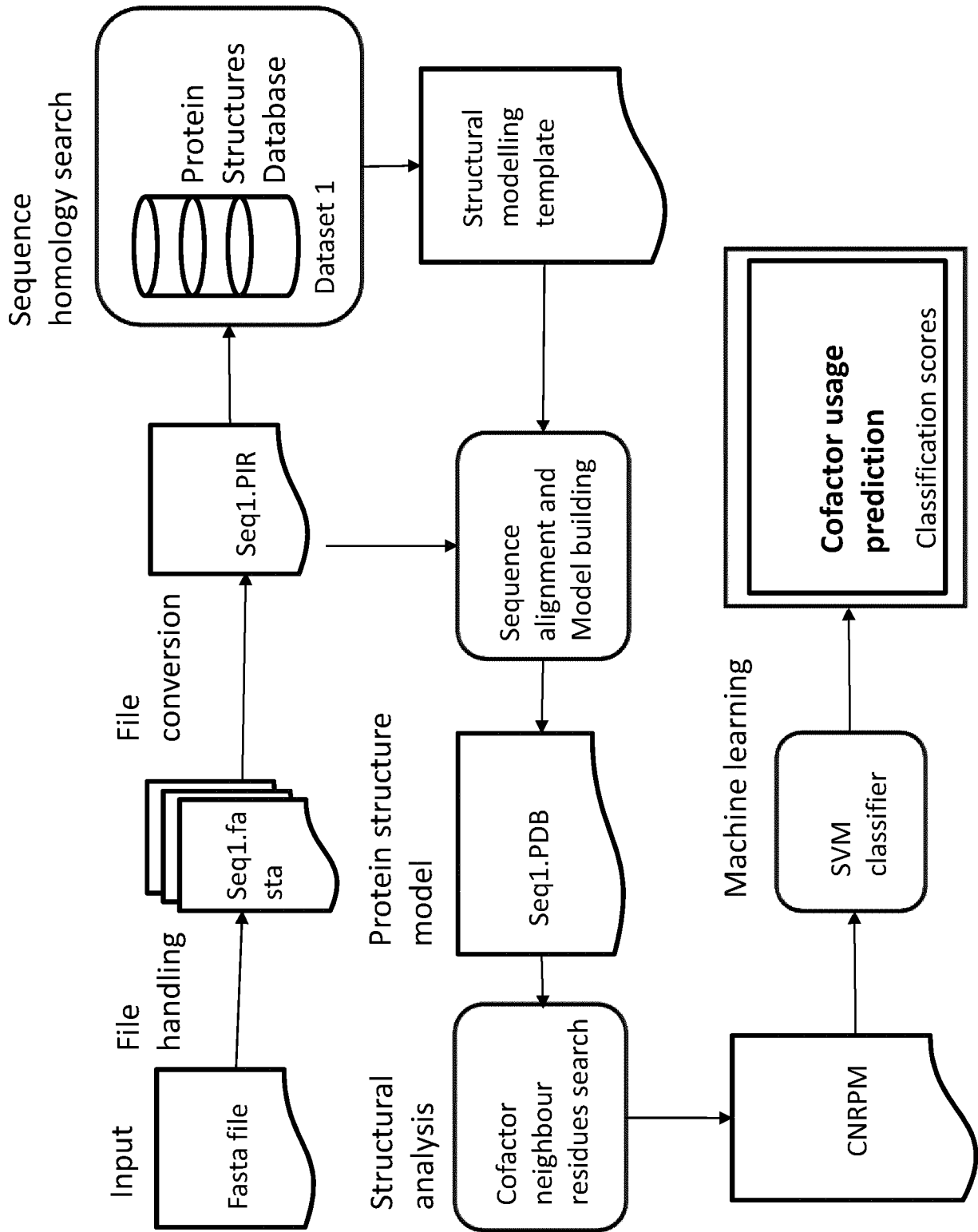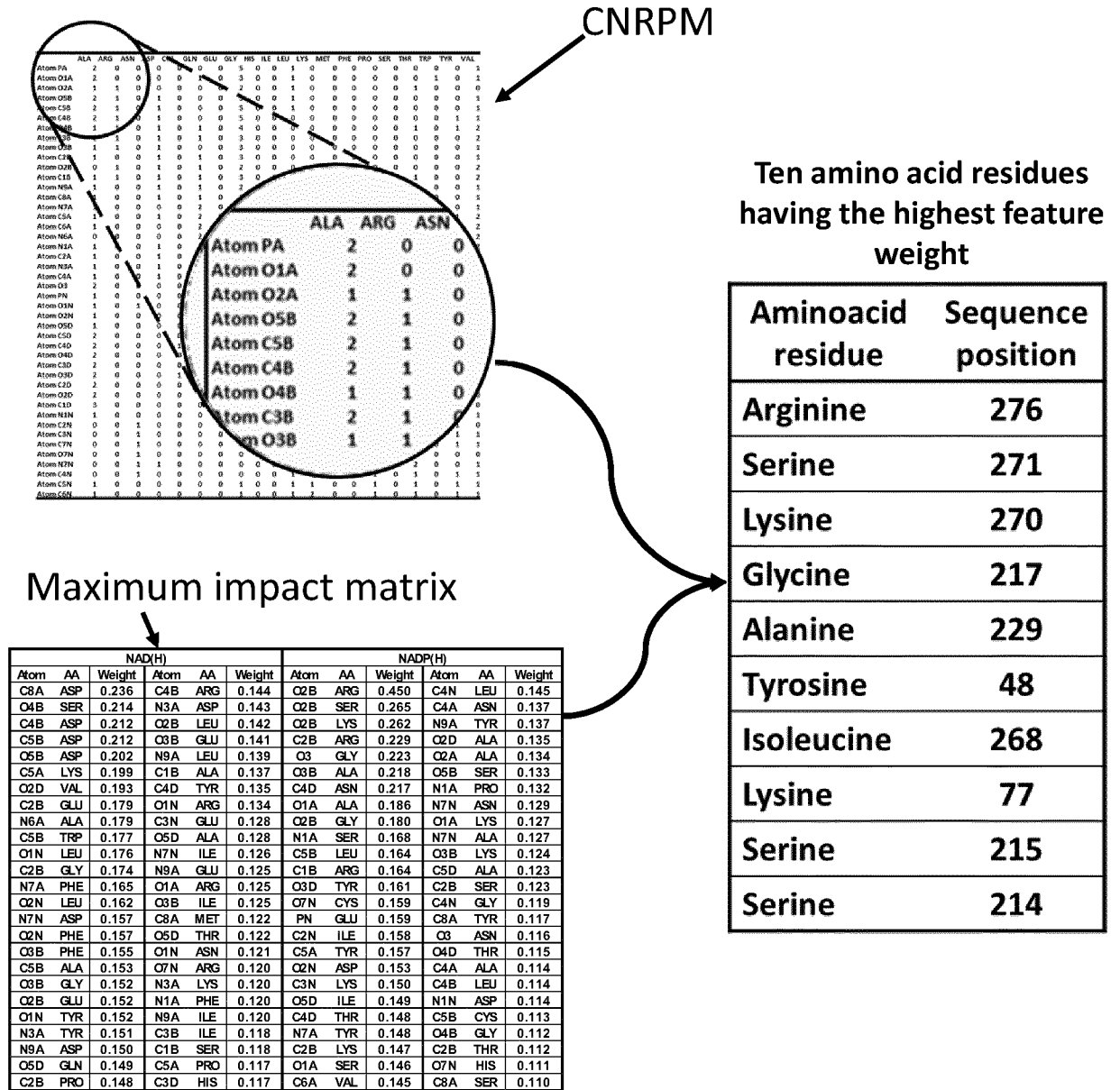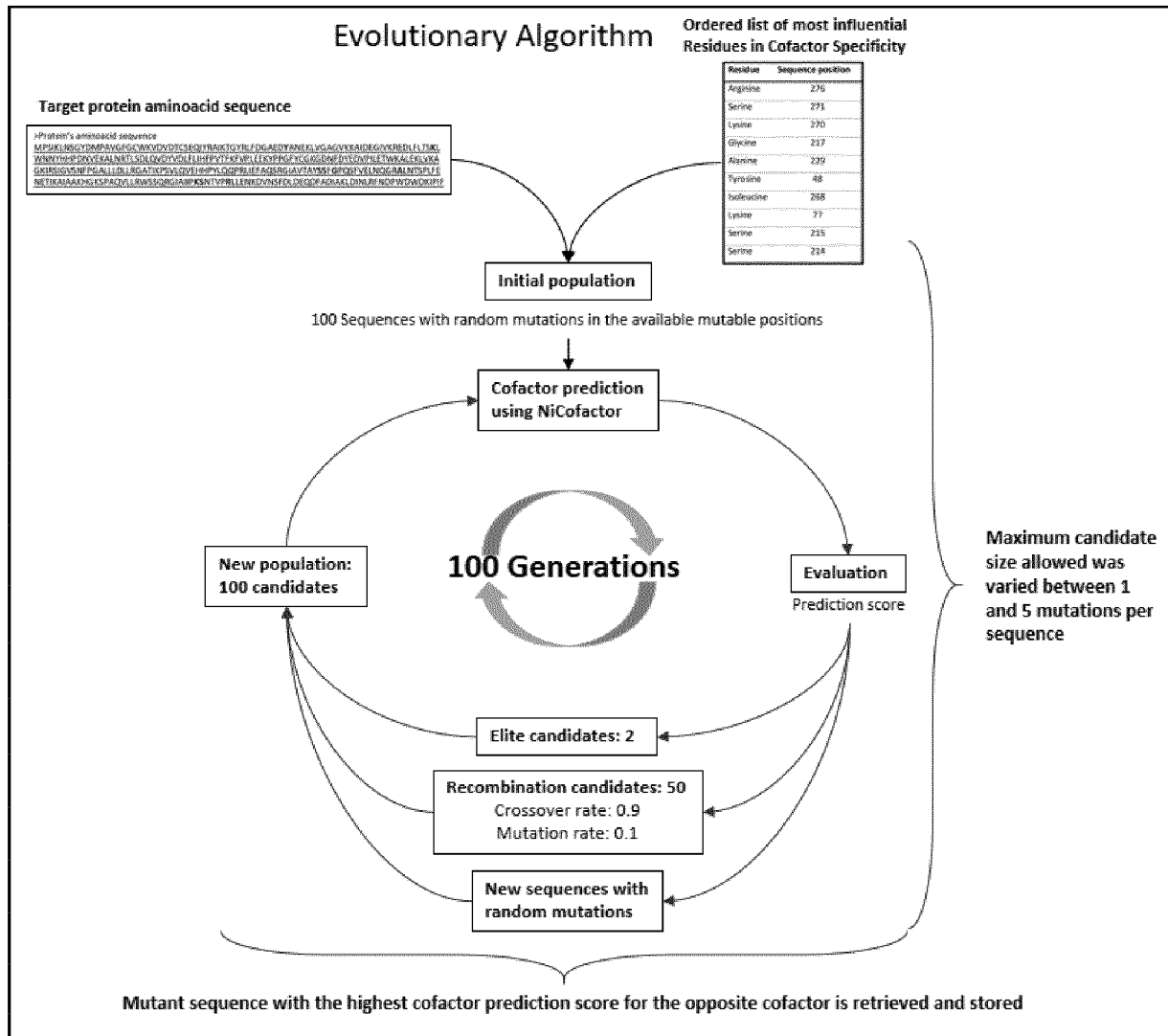| NAD(H)						NADP(H)					
Atom	AA	Weight	Atom	AA	Weight	Atom	AA	Weight	Atom	AA	Weight
C8A	ASP	0.236	C4B	ARG	0.144	O2B	ARG	0.450	C4N	LEU	0.145
O4B	SER	0.214	N3A	ASP	0.143	O2B	SER	0.265	C4A	ASN	0.137
C4B	ASP	0.212	O2B	LEU	0.142	O2B	LYS	0.262	N9A	TYR	0.137
C5B	ASP	0.212	O3B	GLU	0.141	C2B	ARG	0.229	O2D	ALA	0.135
O5B	ASP	0.202	N9A	LEU	0.139	O3	GLY	0.223	O2A	ALA	0.134
C5A	LYS	0.199	C1B	ALA	0.137	O3B	ALA	0.218	O5B	SER	0.133
O2D	VAL	0.193	C4D	TYR	0.135	C4D	ASN	0.217	N1A	PRO	0.132
C2B	GLU	0.179	O1N	ARG	0.134	O1A	ALA	0.186	N7N	ASN	0.129
N6A	ALA	0.179	C3N	GLU	0.128	O2B	GLY	0.180	O1A	LYS	0.127
C5B	TRP	0.177	O5D	ALA	0.128	N1A	SER	0.168	N7N	ALA	0.127
O1N	LEU	0.176	N7N	ILE	0.126	C5B	LEU	0.164	O3B	LYS	0.124
C2B	GLY	0.174	N9A	GLU	0.125	C1B	ARG	0.164	C5D	ALA	0.123
N7A	PHE	0.165	O1A	ARG	0.125	O3D	TYR	0.161	C2B	SER	0.123
O2N	LEU	0.162	O3B	ILE	0.125	O7N	CYS	0.159	C4N	GLY	0.119
N7N	ASP	0.157	C8A	MET	0.122	PN	GLU	0.159	C8A	TYR	0.117
O2N	PHE	0.157	O5D	THR	0.122	C2N	ILE	0.158	O3	ASN	0.116
O3B	PHE	0.155	O1N	ASN	0.121	C5A	TYR	0.157	O4D	THR	0.115
C5B	ALA	0.153	O7N	ARG	0.120	O2N	ASP	0.153	C4A	ALA	0.114
O3B	GLY	0.152	N3A	LYS	0.120	C3N	LYS	0.150	C4B	LEU	0.114
O2B	GLU	0.152	N1A	PHE	0.120	O5D	ILE	0.149	N1N	ASP	0.114
O1N	TYR	0.152	N9A	ILE	0.120	C4D	THR	0.148	C5B	CYS	0.113
N3A	TYR	0.151	C3B	ILE	0.118	N7A	TYR	0.148	O4B	GLY	0.112
N9A	ASP	0.150	C1B	SER	0.118	C2B	LYS	0.147	C2B	THR	0.112
O5D	GLN	0.149	C5A	PRO	0.117	O1A	SER	0.146	O7N	HIS	0.111
C2B	PRO	0.148	C3D	HIS	0.117	C6A	VAL	0.145	C8A	SER	0.110

Fig. 4

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2019/083950

A. CLASSIFICATION OF SUBJECT MATTER

INV. G16B15/30 G16B15/20
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G16B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, COMPENDEX, EMBASE, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Jackson Cahn ET AL: "Cofactor Specificity Reversal Structural Analysis and Library Design", , 1 December 2015 (2015-12-01), page 15, XP055593786, Retrieved from the Internet: URL:http://www.che.caltech.edu/groups/fha/ CSRSALAD/CSRSALAD_Documentation_Dec15.pdf page 3, paragraph 1 page 4, paragraphs 2,3 Sections 4.2.2, 4.2.2.1, 4.2.2.2 4.2.3.1 ----- -/--	1-14

|X| Further documents are listed in the continuation of Box C. |X| See patent family annex.

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7 February 2020	18/02/2020

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C(Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	
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A	NICKOLAY A. KHAZANOV ET AL: "Exploring the Composition of Protein-Ligand Binding Sites on a Large Scale", PLOS COMPUTATIONAL BIOLOGY, vol. 9, no. 11, 21 November 2013 (2013-11-21), page e1003321, XP055593801, DOI: 10.1371/journal.pcbi.1003321 the whole document in particular Table 4. -----	1-14
A	Cláudio Soares: ""Using strucutral information for predicting NAD(P)(H) cofactor specifi" by Tiago Resende, Isabel Rocha et al.", , 28 September 2017 (2017-09-28), XP055593860, Retrieved from the Internet: URL:https://dc.engconfintl.org/enzyme_xxiv /75/ [retrieved on 2019-06-04] the whole document -----	1-14

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