1. SUMMARY

Program focus. *Understanding the biological role and function of molybdoenzymes and their impact on health.*

Overarching direction. Molybdoenzymes (**Mo-enzymes**) play critical roles in bacterial metabolic diversity, global geochemical cycles, and human health. At the Mo-enzyme catalytic core lies the mononuclear molybdenum cofactor (**Moco**, **Table 1),** which comprises a Mo atom coordinated by a complex heterocyclic moiety known as pyranopterin (**PPT**). The current paradigm for Mo-enzyme research focuses on the Mo atom and its immediate environment, *but this approach has failed to explain the extraordinary diversity of Mo-enzyme substrates*. My research program will test the hypothesis that the PPT is critical in controlling catalysis [1, 2]. Mo-enzymes often also contain chains of other cofactors which allow long-range (~100 Å) electron-transfer. Mo-enzymes are also often membrane bound. I will address the following fundamental questions. (*i*) *What is the role of the pyranopterin in defining molybdoenzyme substrate specificity?* (*ii*) *What is the relationship between the Moco and its electron-transfer relay?* (*iii*) *Is there link between Mo-enzyme content and bacterial pathogenicity?* (*iv*) *Can insights into Mo-enzyme structure and function be translated into therapies for human Mo-enzyme deficiency?*

Advancement of knowledge and implications for healthcare. Mo-enzyme deficiency has consequences ranging from chronic to catastrophic, and manifests itself in two ways: (*i*) deficiency of function due to point mutations in the respective structural genes; and (*ii*) generalized deficiency due to mutations in the genes encoding the Moco biosynthetic pathway. Deficiency is associated with two enzymes: xanthine dehydrogenase (**XDH**) and sulfite oxidase (**SUOX**). XDH deficiency results in xanthine accumulation as tissue deposits and the formation of kidney stones, which can lead to acute renal failure. SUOX deficiency has catastrophic consequences due to protein sulfitolysis, causing death in early infancy. Another link between Mo-enzymes and human health is the observation that the genomes of bacterial pathogens tend to encode few or none of them [2], which may open avenues leading to new antimicrobial agents. *Although my program does have implications for human health, my vision is unashamedly of basic science discovery. "It's simple: If there is no basic research upstream, there is no application and therefore no added value downstream." Dr. Alain Beaudet, CIHR President, June 2013.*

Core expertise and infrastructure. My lab has expertise and infrastructure to support the following core techniques: molecular genetics [3–6]; protein overexpression and purification (soluble and membrane-bound [7, 8]); rapid reaction kinetics [9–11]; bioinformatics, proteomics and structural biology [1, 2, 4, 12–15]; fluorescence, optical and electron paramagnetic resonance spectroscopies [3, 4, 16–20]. Where the necessary expertise is lacking in-house, I utilize the collaborations described in **Section 3A**.

Mentoring. I provide a training environment for metalloenzyme research that is without equal in Canada. I was the founding member of the CIHR Membrane Protein Disease Research Group, which is Canada's premier consortium addressing the interplay between membrane proteins and disease. My trainees also have access to an outstanding range of international collaborators. Overall, they will be well-prepared to address the biochemical and biomedical research challenges of the 21st century.

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2A. RESEARCH CONCEPT

Electron-transfer in Mo-enzymes: from basic science to human health. Electron-transfer is essential for the process of oxidative phosphorylation, whereby electrons from highly reducing compounds such as NADH are transferred to oxygen, leading to transmembrane proton translocation and synthesis of ATP. In archaea and bacteria, archetypal oxidative phosphorylation is complemented by a diversity of electron-transfer reactions, permitting growth on a wide range of reducing and oxidizing substrates under both aerobic and anaerobic conditions. Electron-transfer reactions also participate in global geochemical cycles and environmental homeostasis. Aside from their primary role in energy conservation, electron-transfer reactions play roles in processes such as purine metabolism and sulfur homeostasis, which directly impact human health. Mo-enzymes are an important class of electron-transfer enzymes, which catalyze at least 17 distinct types of redox reactions spanning a reduction potential ($\boldsymbol{E_m}$) range exceeding 1Volt [2, 12, 13]. *I seek an understanding of how Mo-enzymes achieve this remarkable flexibility, and I will apply this knowledge to address issues such as human Mo-enzyme deficiency and bacterial pathogenicity.*

The foundation of my program is a career spent studying electron-transfer enzymes containing a range of cofactors: hemes [19, 21, 22], iron-sulfur clusters [4, 18, 23, 24], flavins [25–27], and the mononuclear molybdenum cofactor (**Moco**) [1, 16, 28, 29]. As described below, developing an understanding of Mo-enzymes has been hampered by a focus on the metal and its immediate environment [2]. I will shift this metallocentric paradigm and address the role of the non-metal component of the cofactor known as pyranopterin (**PPT**, **Figure 1**)**.** This will lead to a complete understanding of how PPT functions in defining Mo-enzyme substrate diversity.

My overarching program goal is to generate an atomic-resolution understanding of how Mo-enzymes function and their role in human health.

Research Objectives. To achieve my overarching goal, I will test the following hypotheses.

- ***i. The organic component of the molybdenum cofactor known as pyranopterin can exist in multiple oxidation states that are critical to molybdoenzyme substrate specificity.***
- ***ii. The coordination environment of the pyranopterin modulates metal redox chemistry and reactivity.***
- ***iii. New insights into bacterial pathogenicity will be revealed by the generalized lack of Mo-enzymes encoded by pathogen genomes.***
- ***iv. Multiplicity of Mo-enzymes in the gut bacteria of Crohn's disease patients will provide insights into to the cause of this debilitating condition.***

The Swiss army knife of metabolic diversity. The catalytic heart of Mo-enzymes comprises a Mo atom coordinated by one or two PPT dithiolene chelates (**Figure 1**). PPT is a tricyclic heterocycle comprising pyrimidine, piperazine, and pyran rings (labeled **a**, **b**, and **c** in **Figure 1A**). Protein crystallography has permitted Mo-enzyme assignment to three major families distinguished by their overall protein folds [2]. *Among cofactors, Moco exhibits the unique property of scalable complexity, as illustrated by its structure in the following three major families of Mo-enzymes*. (*i*) The **SUOX-fold** family, which includes bacterial, plant, and mammalian sulfite oxidases, and plant assimilatory nitrate reductase [12]; these contain the simplest Moco – the **mo**lybdo-**p**yrano**pt**erin (**Mo-PPT**) illustrated in **Figure 1A**. (***ii***) The xanthine dehydrogenase (**XDH-fold**) family, which participates in a range of reactions including oxidation of purines, isoquinoline, nicotinic acid (vitamin B_3) and carbon monoxide. While eukaryotic and some prokaryotic XDH-fold enzymes contain Mo-PPT, most prokaryotic examples contain the more complex **mo**lybdo-**p**yranopterin **c**ytosine **d**inucleotide (**Mo-**

PCD) illustrated in **Figure 1B** [2]. The aerobic carbon monoxide dehydrogenases have additional modifications, including the presence of a dinuclear heterometal active-site (CuSMo(=O)OH) (*inset*, **Figure 1B**) or incorporation of a selenium atom at the active site (as a Mo-S-Se moiety) [30]. (***iii***) The dimethylsulfoxide reductase (**DMSOR-fold**) family, of which the eponymous member is the *Rhodobacter* dimethylsulfoxide reductase [13]. These typically contain the **mo**lybdo-**bis**(**p**yranopterin **g**uanine **d**inucleotide) (**Mo-bisPGD**) illustrated in **Figure 1C**. A consequence of the scalable complexity of the Mo-pyranopterin cofactors is their relatively enormous biosynthetic cost. Up to ten gene products are necessary for their biosynthesis [31], raising the critical question: w*hat is the role of the non-metallic component of the cofactor?*

Mo-enzymes and human health. Mo-enzymes impact human health in three ways. (*i*) Deficiency of function, arising either from Moco deficiency or from point mutations in Mo-enzyme structural genes, can have serious consequences. (*ii*) Mo-enzymes tend to be absent from pathogens, but paradoxically play a role in defining the gut microbiome of patients with the debilitating inflammatory bowel disease known as Crohn's disease. (*iii*) Mo-enzymes play roles in global carbon, sulfur, and nitrogen cycles, and therefore play a role in environmental homeostasis that indirectly impacts human health.

***Mo-enzyme deficiency: from chronic to catastrophic*.** The most prominent example of Mo-enzyme deficiency is that of sulfite oxidase (**SUOX**), which functions in the breakdown of cysteine and methionine. Deficiency, either via enzyme-inactivating point mutations [32] or via mutations in the Moco biosynthesis pathway [33], typically results in death in early infancy. Lethality is due to intracellular sulfite build up, which causes sulfitolysis of disulfide bonds resulting in protein instability and subsequent degradation [34]. Symptoms include neurodegeneration, intractable seizures, mental retardation, and ocular lens dislocation [35]. In the case of Moco deficiency, there is a therapy that comprises regular injections of the biosynthetic intermediate cyclic pyranopterin monophosphate [36]. *However, there remains no treatment for SUOX deficiency due to enzyme-inactivating point mutations.* In the case of xanthine dehydrogenase (**XDH**) deficiency, the consequences are more chronic than catastrophic, resulting in xanthine accumulation as tissue deposits and as urinary tract calculi (kidney stones), which in extreme cases can lead to acute renal failure [35]. XDH deficiency (xanthinuria) treatments include high fluid intake, dehydration prevention, and consumption of a low purine diet. *The foundation of future breakthroughs in treating patients with Mo-enzyme deficiency will be a clearer atomic-resolution understanding of how these enzymes function.*

The Mo-enzyme pathogenicity paradox. My work on the taxonomic distribution of Mo-enzymes led to the following observations [2, 12, 13]. (*i*) Pathogens tend to lack Mo-enzymes and thus exhibit more limited metabolic competency than non-pathogens (**Figure 2**). *Detailed examination of this lack of Mo-enzymes will yield new insights into the mechanisms of bacterial pathogenicity and may reveal new targets for antimicrobial agents*. (*ii*) The gut flora of Crohn's disease patients includes species of bacteria with *unusually high numbers of Mo-enzymes, particularly those of the DMSOR-fold family*. One example is *Gordonibacter pamelaeae* [37], whose proteome is predicted to comprise 49 such enzymes, including 26 acetylene hydratases and 8 enzymes, predicted to act on *S*- and *N*- oxide substrates. Another example is *Eggerthella lenta* [38, 39], whose proteome is predicted to comprise 34 Mo-enzymes, 11 of which are predicted to act on *S*- and *N*- oxide substrates, and 8 of which are predicted to catalyze sulfur anion reduction. *I predict that the presence in the gut of bacteria with high Mo-enzyme multiplicity is a symptom rather than a cause of Crohn's disease, reflecting an altered gut chemical composition that may reveal its true cause.* I will exploit the relationships of Mo-enzymes with both bacterial pathogenicity and Crohn's disease with the objective of identifying new antimicrobial agents, biomarkers, and disease therapies.

Reinvigorating the field – shifting the metallocentric Mo-enzyme paradigm. Attempts to understand Mo-enzymes have focused directly on their active sites and specific interactions between the Mo atom coordination sphere and the substrate, as well as substrate-active site amino acid interactions [30, 40, 41]. This narrow focus has hindered progress, with a large number of papers generating only incremental advances. In 2012, I published the observation that the PPT itself can exist in multiple oxidation states and may even undergo redox-cycling during catalysis (**Figure 3**) [1]. This provided the first convincing explanation for how Mo-enzymes can catalyze such an extraordinary variety of redox transitions. *I presented the hypothesis that the PPT environment determines its oxidation state, and that this in turn redox-tunes the Mo atom facilitating the observed substrate diversity.* In the case of the SUOX-fold enzymes, my ability to look beyond the Mo atom led to the discovery of a charge-transfer relay comprising conserved Tyr and His residues that connects the sulfite binding site to the piperazine ring of the PPT [2] (**Figure 4**). *Using a combination of molecular genetics, biophysics, and structural biology, I will examine the role of the PPT coordination environment in defining Mo-enzyme function.*

Tractable model systems. Testing my hypotheses requires the use of rapidly modifiable model enzyme systems that can be readily over-expressed and evaluated. For this reason, I have *initially* chosen three well-characterized enzymes of known structure that are already well-established model systems in my laboratory: two members of the DMSOR-fold family (*Escherichia coli* nitrate reductase [NarGHI] [4, 7, 18–20, 22] and DMSO reductase [DmsABC] [5, 6, 16]); and a member of the SUOX family (*E. coli* YedY [8, 42–44]). I will develop additional model systems as needed.

Significance and Impact of program outputs. My program will reinvigorate Mo-enzyme research by addressing the following questions. *How has evolution enabled effective electrochemistry and electron-transfer? How does the PPT contribute to catalysis? What are the consequences of disease-mimicking variants on catalysis? What is the link between Mo-enzyme multiplicity and Crohn's disease? Will the lack of Mo-enzymes in pathogens reveal new targets for antimicrobial agents?*

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2B. RESEARCH APPROACH

The role of PPT coordination. This will be addressed by designing appropriate variants, and characterizing them using electron paramagnetic resonance (**EPR**), fluorescence spectroscopy, kinetic methods, novel electrochemical methods (e.g. protein film voltammetry, **PFV** [45]), and protein crystallography. *In this way, I will construct a detailed model for how PPT coordination defines Mo-enzyme function*.

Human disease. My examination of the predicted proteomes of bacteria within the gut microbiome of Crohn's disease patients revealed the presence of large numbers of Mo-enzymes [2]. I will collaboratively address this by analyzing the fecal chemical composition of Crohn's patients. I predict the measurement of high concentrations of Mo-enzyme substrates, including *S*- and *N*-oxides and their respective sulfides and amides. Molecular genetics techniques will be used to express selected enzymes from Crohn's disease-related bacteria in *E. coli*, and their substrate specificities will be evaluated.

Tractable model systems. Risk will be mitigated in my PPT coordination work by initially using 3 tractable model systems, each having distinct advantages. (*i*) ***NarGHI*** is a membrane-bound heterotrimer which can be overexpressed to spectroscopic purity in the *E. coli* inner membrane, and is also amenable to protein crystallography [7]. It is a mature and predictable model system, and I have already generated a significant literature on the interplay between its protein structure and its Moco (which is a **Mo-bisPGD**, **Figure 1C**) [4, 18]. (*ii*) ***DmsABC*** is also a membrane-bound heterotrimer that can be overexpressed to high spectroscopic purity within the *E. coli* inner membrane [5, 6]. *Because it acts on a broad range of S- and N-oxides, it is a useful system for exploring the etiology of Crohn's disease.* Like NarGHI, it also contains a Mo-bisPGD cofactor, allowing hypothesis testing to be extended over the two systems. (*iii*) ***YedY – the ultimate minimalist Mo-enzyme –*** is emerging as a classic model system for studies of both the molybdenum and the PPT [8, 42, 43, 46]. YedY is the simplest known SUOX-fold enzyme and contains a Mo-PPT (**Figure 1A**) with no additional cofactors, allowing spectroscopic scrutiny without interference from non Mo-PPT centers. *I will use YedY to demonstrate that altering the PPT coordination environment modulates Mo electrochemistry and substrate reactivity*.

Methods. I will rapidly generate, overexpress and purify enzyme variants. These variants will then be characterized by spectroscopic and electrochemical methods (e.g. potentiometric titrations followed by EPR). This is complemented by expertise in structural biology and bioinformatics [1, 2, 13, 13]. Completion of hypothesis-testing will also require expertise from other research groups (e.g. protein crystallography, novel electrochemical methods [e.g. PFV], theoretical chemistry, magnetic circular dichroism [**MCD**], X-ray absorption spectroscopy [**XAS**], gastroenterology). My collaborations are listed in **Section 3A**). *Overall, I have established a robust work flow that when necessary can utilize outside expertise* [1, 7, 8, 42, 43].

Challenges and their mitigation. Challenges almost always stem from a lack of in-house access to cutting-edge techniques. Their mitigation involves either acquiring the additional research infrastructure (**Section 3C**), or collaborating with investigators who wield the necessary techniques (**Section 3A**). This has been my successful approach over almost 40 years as an independent investigator.

Flexible strategies to ensure success. The following examples illustrate how I will expedite progress. (*i*) **NarGHI – PPT coordination controls catalysis.** Analyses of known Mo-enzyme structures revealed the importance of PPT-coordination in defining enzyme function and diversity of substrate specificity [2]. **Figure 5** shows the Mo-bisPGD cofactor of NarGHI and the positions of conserved His residues in the vicinity of the piperazine rings of its Mo-bisPGD. The His residue that bridges the two

piperazines is replaced by an Arg in the formate dehydrogenases (a sub-family of DMSOR-fold enzymes), and the other conserved His residue is replaced by a Gln in a subset of the formate dehydrogenases. These differences may explain how NarGHI is able to reduce nitrate (E_m=>+400mV), whereas the closely-related formate dehydrogenase is able to oxidize formate (E_m=<-400mV). Variants with substitutions of these residues will be generated, and their effects on Mo electrochemistry determined by EPR spectroscopy (e.g. as in [4, 5, 44]). As appropriate, other methods will be used (e.g. protein crystallography, theoretical chemistry, PFV). I will extend hypothesis testing to DmsABC, to confirm that PPT coordination has a role in defining Mo electrochemistry in the DMSOR-fold enzymes. *I will show how precisely the PPT coordination environment controls Mo-enzyme.* (*ii)* **YedY – direct measurement of PPT redox transitions.** Unlike other members of the SUOX-fold family, YedY lacks heme and Mo-PPT is its only cofactor [2]. YedY is therefore uniquely amenable to techniques such as EPR [44], XAS [42], MCD, and PFV. I am using YedY to test the hypothesis that SUOX-fold enzymes contain a partially-oxidized form of the PPT (the 10,10a-dihydro form, **Figure 3**), and reasoned that the 10,10a-dihydro to tetrahydro transition should be visible in PFV experiments. *I have demonstrated the existence of reactions consistent with the hypothesized 10,10a-dihydro to tetrahydro transition.* I am capitalizing on this by using enzyme variants designed to modulate the observed transition to show how it impacts Mo electrochemistry and substrate reactivity. Once completed, this work will revolutionize our understanding of Mo-enzyme function. (*iii*) **Crohn's disease**. I will collaboratively analyze lower intestine contents to establish chemical differences between healthy and diseased individuals. Enzyme systems from bacteria associated with Crohn's disease will expressed in *E. coli* via codon-optimized *de novo* gene synthesis and their substrate specificities evaluated. (*iv*) ***Bacterial pathogenicity*.** The sheer breadth of pathogenic organisms renders addressing them individually in the context of Mo-enzyme content impractical. I will continue my successful bioinformatics strategy with the aim of dovetailing this with a systems biology metabolic modeling approach to identify new targets for antimicrobial agents.

Measurements of successful progress. I will continue to maintain a high tempo of research outputs with an emphasis on publication in high-ranking international journals (see **CCV**) and participation in international meetings such as the prestigious European Bioenergetics Conferences, Gordon Research Conferences, and Biophysical Society Meetings. Another measure of success is my continuing success in the training of highly qualified personnel (**Section 3B**).

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3A. EXPERTISE

My research program exploits expertise and infrastructure that I have developed at the University of Alberta over almost 40 years. I am recognized as an international leader in the study of bacterial electron-transfer enzymes, emphasizing those that contain Moco. I have published over 200 papers (see **CCV**), all of which have been in high-impact international journals, and I am a frequent contributor to top-tier journals, including *Cell*, *PNAS*, *Nature Biotechnology, Nature Chemical Biology*, and *Nature Structural Biology*.

Research Infrastructure. I have established metalloenzyme research infrastructure that is without equal in Canada. I obtained funding for an electron paramagnetic resonance facility able to characterize species ranging from semiquinone radicals, iron-sulfur clusters and hemes, to the Moco that is central to my research program. My lab contains Bruker Elexsys E500 and ESP300E EPR spectrometers that are unique in Canada in being able to record spectra at any temperature between 4.2K and 350K. I also have multiple fermenters (B. Braun Biostats) for cell growth and protein overexpression, equipment to perform large scale cell lysis (e.g. an Avestin Emulsiflex C3) and protein purification (e.g. an ÄKTA FPLC), as well as a range of optical spectrometers (ultraviolet, visible, and fluorescence) and an Applied Photophysics SX-17MV stopped-flow spectrophotometer.

Emerging techniques. *My success is based on a willingness to embrace emerging techniques.* These include, in approximate order of application: use of bacterial plasmids (1970s); DNA cloning, sequencing, and overexpression (1980s); enzyme tagging, affinity chromatography, protein purification and crystallography (1990-present); spectroscopic and kinetic techniques such as electron paramagnetic resonance (EPR) and stopped flow kinetics (1980-present); and "informatics"and "omics" driven endeavors (2000-present).

Recognition of effective leadership. My expertise has attracted worldwide recognition. I have presented >100 talks at international conferences and other universities, including at Gordon Research Conferences and European Bioenergetics Conferences. I have also had leadership roles in the organization of 21 conferences, most notably being the President of the 19th International Congress of Biochemistry and Molecular Biology (2003) and the Chair of the International Meeting on Mo-enzymes (2011). Recent awards recognizing my accomplishments include a Canada Research Chair in Membrane Biochemistry (2001-2008), a Killam Annual Professorship (2005-2006), an International Union of Biochemistry and Molecular Biology Distinguished Service Award (2006), and election as a Fellow of the Royal Society of Canada in 2011 (for others, see **CCV**). *These awards recognize my ability to generate and disseminate very high quality contributions and my ability to communicate my work at prestigious international meetings.*

Institutional and international synergies. Scientific progress requires collaborations between individual lab members, as well as the construction of multidisciplinary synergies between research groups. One of many examples of my ability to foster collaborations is my formation of, and leadership of, the CIHR Membrane Protein Research Group in 1990. Initially this comprised 5 research teams at the University of Alberta, and immediately became an incubator that matured a large number of collaborations into prestigious publications. Today, this group is known as the CIHR Membrane Protein Disease Research Group, and comprises 12 teams.

My expertise has been pivotal in overcoming the challenges of membrane protein overexpression, particularly in multi-cofactor systems. To address the problems of NarGHI over-expression, I led a NATO-funded collaborative program with the *Centre National de la Recherche Scientifique* in Marseille, working with Drs. Gérard Giordano and Francis Blasco, leading to many of the protein overexpression and characterization methodologies that will be used in my research

program. Our success rendered NarGHI an attractive target for membrane protein crystallography, which at that time (late 1990s) was a field fraught with challenges. Undaunted, I assembled an international team of researchers (including my Marseille collaborators and Natalie Strynadka [UBC]), with the aim of establishing methods to purify large quantities of highly purified NarGHI and protocols for its crystallization, leading to crystals that diffracted at an outstanding 1.9Å resolution. I recruited another team member (Fraser Armstrong of Inorganic Chemistry Laboratory, Oxford) to apply novel electrochemical techniques (such as PFV) to NarGHI with the aim of understanding the kinetics of electron-transfer through its cofactors. This project was funded by the Human Frontiers Science Program Organization.

To complete the structure of NarGHI, I obtained NIH funding that also allowed me to broaden the number of molybdoenzyme targets for protein crystallography. My structure of NarGHI was published in *Nature Structural Biology* in 2003, and throughout the term of our NIH funding we exploited our collection of enzyme variants to generate high-impact publications addressing NarGHI function (e.g. quinol binding) and maturation (e.g. Moco insertion, function, and enzyme maturation). One of our additional targets for crystallography was the *E. coli SUOX fold enzyme* YedY, which belongs to a vast subfamily of enzymes comprising the majority of those with the SUOX protein fold. Our 2.2Å resolution YedY structure, along with our characterizations of its electrochemistry, have led it to becoming an ideal model system for studies of the Mo-PPT cofactor.

I have also played a leadership role in university administration, most notably as Associate Dean for Research in the Faculty of Medicine (1993 – 2005), demonstrating my ability to balance the roles of administrator and scientist. *Overall, I clearly have the appropriate expertise and experience to lead the proposed research to successful outcomes.*

***Role of highly qualified personnel and technicians*.** I have always recognized the need to have ongoing expertise in the lab to maintain my research infrastructure and to provide technical training to students. With this in mind, my program will utilize a senior research associate with long-standing expertise in cryogenic EPR, and a technologist with extensive experience in cell culture, molecular genetics, and protein purification

Synergies with other research groups. My collaborators are enthusiastically committed to my program, and provide expertise in challenging (e.g. crystallography) or emerging techniques (e.g. Fourier transform PFV). Collaborations relevant to this proposal include the following:

Natalie Strynadka (UBC). *Protein crystallography of selected variants; completion of hypothesis testing often requires atomic-resolution structural insights.* Where necessary, high-resolution protein structures generated with established methodologies will simplify data interpretation, for example where we need to be absolutely sure that variant enzymes do not lack critical cofactors. In addition, my interest in newly-discovered Mo-enzymes will generate new targets for protein structure determination. Dr. Strynadka has critical expertise in generating structures of membrane proteins.

Martin Kirk (University of New Mexico). *Theoretical chemistry, magnetic circular dichroism* (**MCD**). Martin Kirk focuses on studies of model compounds that mimic the molybdenum cofactor, with the aim of generating an understanding of the role of the PPT in modulating substrate reactivity. My discovery and characterization of YedY spawned a research avenue that parallels Dr. Kirk's model compound work. Dr. Kirk will use MCD to study the structure of the Mo coordination sphere. He will also apply theoretical chemistry to rationalize changes in the PPT coordination sphere at the quantum mechanical level (in YedY, DmsABC, and NarGHI).

Alison Parkin (University of York, UK). *Shedding light on Mo-enzymes using novel electrochemical methods.* Dr. Parkin trained with Fraser Armstrong at the Inorganic Chemistry Laboratory at the

University of Oxford. She will use her expertise in Fourier transform PFV to directly measure Mo-enzyme redox transitions, and will also apply spectroelectrochemical and resonance Raman techniques to explore PPT oxidation states.

Graham George (University of Saskatchewan). *Using XAS to probe the Mo coordination environment.* Dr. George has access to the Canadian Light Source XAS beam line, and will use this to evaluate the metal coordination environment of Mo-enzymes to reveal exquisite details of ligand identity, number, and metal-ligand distance in a way that is impossible to do at available resolutions of protein crystal structures. We are using XAS to investigate the effects of variants of PPT-coordinating amino acids on the metal coordination sphere – a prime example being the ability of XAS to distinguish between dithiol and thiol-thione coordination in variants containing PPT in the dihydro oxidation state.

Edith Vacell (University of Alberta). *Evaluating the link between fecal chemical composition and Crohn's disease.* Dr. Vacell trained with Stephen Vanner at the Gastrointestinal Diseases Research Unit at Queen's University, and is an accomplished physician-scientist. She is an expert on Crohn's disease and has an interest in bacterial pathogenicity with an emphasis on infections of the lower gastrointestinal tract. Dr. Vacell's patients will provide a wealth of information on fecal chemical composition in her Crohn's disease patients. Dr. Vanner's knowledge of the clinical literature will guide my analyes of bacterial proteomes and aid in selection of Mo-enzymes for expression in *E. coli.*

My expertise is primarily multidisciplinary, based on the experience that true paradigm shifts can only be achieved using a diversity of applicable techniques. To move forwards, we must recognize and acquire new techniques in-house. Where we cannot acquire the necessary new techniques, we must build synergies with those who wield them.

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3B. MENTORSHIP AND TRAINING.

Perhaps the biggest challenge facing Canada in the 21st century is its chronic shortage of highly skilled scientists and engineers. The University of Alberta is addressing this by aggressively investing in research infrastructure, cementing its position as an exceptionally high-ranking institution, and by recruiting world-class faculty and students. My mentorship philosophy dovetails with this institutional ambition, and has generated an exceptional pool of highly qualified personnel. I encourage trainees to take ownership and responsibility for their projects, most specifically in the areas of authorship and communication.

Institutional influences. Trainees are offered an exceptional learning environment at the institutional, faculty, and departmental levels. They are evaluated on their ability to communicate emerging concepts in the literature via two strategies relating to emerging high-impact papers: participation in courses encouraging their critical evaluation through group discussions, and in courses requiring journal club presentations of such papers chosen by the trainees. Students are also required to present both their own work and emerging high-impact work on an unrelated topic in a formal lecture format. They also have to complete appropriate graduate-level courses, and a comprehensive candidacy exam. Their progress is monitored by the department via a student-specific supervisory committee. At the postdoctoral level, trainees participate in journal clubs and seminar series, where they can report on their own research progress and productivity.

Direct mentorship. I provide direct mentorship and program direction to my trainees, meeting with them informally on an almost-daily basis, while my research associate and technician provide training in the use of specific techniques and analysis of the data derived therefrom. Trainees present their results semi-formally during weekly meetings of my group.

Group membership influences. The CIHR Molecular Biology of Membranes Research Group (**MPDRG**) has 12 faculty members, and plays a critical role in my mentorship program: (*i*) it has a collective skill-set in membrane protein biology that is without equal in Canada, providing my trainees with an additional vault of expertise; and (*ii*) it provides an expert group to which new ideas and publishable aliquots of data can be presented in regular semi-formal presentations, providing trainees with valuable experience in how to frame their research in a way that renders it accessible to a broader spectrum of biochemists. Trainees present their work at the weekly MPDRG meetings at least once per year.

The MPDRG has also obtained NSERC funding for their prestigious International Research Training Group (IRTG) in Membrane Biology, which provides opportunities and funding for trainees to spend time in the laboratories of German investigators (50% of my trainees will participate). IRTG also provides professional training for research trainees who wish to transition to non-academic career paths, such as those in research administration, technology commercialization, biotechnology companies, and public science policy.

Collaborator influences. An important aspect of my mentorship is exposure of trainees to collaborators. This happens in two ways: students will be sent to work on and learn critical techniques in a collaborator's lab; or a member of a collaborator's lab will visit my group and learn techniques such as EPR, fluorescence spectroscopy, or protein purification. This provides the trainee with access to additional expertise, giving them direct experience of how other research groups function. My trainees spend on average 3 months visiting other research groups.

Positioning trainees for future success. Individual, institutional, and national scientific success is not well served by strategies that are solid but conservative. A need exists for HQPs to move in paradigm-shifting directions, to intelligently question and improve on existing dogmas. I have always encouraged

trainees to embark on new directions, and this is reflected in the successful research topics chosen by former trainees who have academic appointments, including:

Bernard Lemire (University of Alberta) investigates the role of the mitochondrial respiratory chain in maintaining cellular health, using *Saccharomyces cerevisiae* and *Caenorhabditis elegans* as tractable unicellular and complex eukaryotic systems, respectively. Dr. Lemire is also using bioinformatics to elucidate the roles of disease-associated mutations of mitochondrial proteins.

Peter Bilous (Eastern Washington University) built on the molecular genetics skill he learned in my laboratory by establishing the RCMP DNA forensics laboratory in Edmonton in the early 1990s. He then returned to academia and teaches forensic examination methodology.

Russell Bishop (McMaster University) studies bacterial cell wall biogenesis with an emphasis on pathogenicity.

Raymond Turner (University of Calgary) investigates membrane protein assembly and targeting, biofilms, and the mechanisms of action of antimicrobial agents.

John Robinson (Memorial University) studies the sea urchin embryo as a model system for embryonic development.

Damaraju Sambasivarao (University of Alberta) investigates the use of single nucleotide polymorphisms in predicting, diagnosing, and prognosing cancers.

In addition to the above individuals, many have gone on to achieve success in medicine, biotechnology industry, technology commercialization, or university administration.

Identifying and mitigating mentorship challenges. The quality of human resources is the most critical factor in determining the success or failure of a project. It is essential to select only the most promising of students and postdocs. At the departmental level, there is a program of graduate student rotation that occurs soon after recruitment, wherein the students can evaluate various labs and projects, and *faculty members can evaluate how these student recruits will dovetail into their research programs*. Another strategy for trainee recruitment is the use of the Alberta Innovates Health Solutions Summer Studentship Program. This is an excellent way of getting a prospective trainee into the lab for a summer, and historically has been the way I identify and recruit most of my graduate students. In the case of postdoctoral fellows, graduate students of collaborators are potential future recruits, and the reciprocal visit strategy outlined above allows evaluation of a candidate's future potential.

Leveraging trainees' future success. *My proposed research will take place in an exciting and dynamic environment, with opportunities for trainees to participate in synergies with other researchers. Most also participate in exchange visits with my international collaborators. They will attend international conferences and they will publish in top-tier journals. My trainees will add to Canada's next-generation of scientific success stories.*

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3C. QUALITY OF SUPPORT ENVIRONMENT

Provision of research infrastructure. The University of Alberta has a strategy to place itself among the top 20 publicly-funded universities by 2020. A critical part of this is the provision of new infrastructure through an ambitious program of construction and renovation. I have been allocated space in the Katz Group Rexall Building, which is designed to comply with international best practices in terms of laboratory design. My group is located in an "open-plan space", which is also used by other groups within the MPDRG, fostering an exciting and synergistic research environment.

Alignment of research program with identified institutional strengths. My program aligns with the following strengths identified in the University of Alberta's academic plan as areas of national and international excellence: membrane molecular biology and protein structure and function (including proteomics). In the 2010 externally-adjudicated research assessment exercise within the Faculty of Medicine and Dentistry, membrane biology, including studies of membrane proteins such as NarGHI and DmsABC, was a top-rated area of excellence.

Scholarships and Fellowships. The province of Alberta provides opportunities for trainees to apply for prestigious scholarships and fellowships through Alberta Innovates Health Solutions. Institutional scholarship programs are also available, including awards from the Killam Trusts, the Queen Elizabeth II Graduate Scholarship, and the 75th Anniversary Graduate Student Award.

Instututional support for postdoctoral fellows. The University of Alberta's Postdoctoral Fellows Office provides robust practical assistance with issues regarding moving to Alberta, Canadian income tax rules, scholarship applications, and professional development.

Provincial/federal support for equipment purchases. Use of new techniques inevitably entails acquisition of new equipment, and existing equipment is often rendered obsolete by technological advances. The province of Alberta has new equipment funding programs that I have exploited over my career, including obtaining funding for an EPR spectrometer (a Bruker ESP300E), a Bruker Elexsys E500 EPR spectrometer (with additional Canada Foundation for Innovation funding), and a variety of spectrometers, fermenters, and an Emulsiflex C3 cell lysis system. As my program progresses, application of emerging techniques will be facilitated by provincially-funded equipment purchases.

Departmental, Faculty, and Institutional Research Services. My department of hosts the Alberta Proteomics and Mass Spectrometry Facility, which offers services such as mass spectrometry and DNA sequencing on a fee-for-service basis, results in a significant cost-saving for my program. In addition, institutional access to at-cost cryogens such as liquid nitrogen significantly reduces the cost of analyzing molybdenum redox transitions by EPR. The department hosts a comprehensive stores outlet (chemicals and laboratory supplies). The department also provides comprehensive computer support, including software development.

Administrative and technical support. The Department of Biochemistry provides administrative support for my program of research, and also expedites scholarship and fellowship applications to various agencies. There are also department-specific graduate student stipends. Post research award administration assistance is also offered at the departmental level.

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CCV – MOST SIGNIFICANT CONTRIBUTIONS

I have spearheaded the use of *E. coli* model systems to develop an atomic-resolution understanding of electron-transfer enzymes, applying a range of expertise that is unique in Canada, including membrane protein overexpression, purification, molecular genetics, fluorescence spectroscopy and electron paramagnetic resonance (EPR). I have also used my expertise to study transmembrane protein export. My most significant contributions are summarized below:

1. Overexpression and Characterization of Membrane-bound Multisubunit, Multicofactor Enzymes (1976-present). I developed protein expression systems for the following membrane-bound electron-transfer enzymes: fumarate reductase (FrdABCD), succinate dehydrogenase (SdhCDAB), DMSO reductase (DmsABC), and nitrate reductase (NarGHI), In the case of FrdABCD and NarGHI, membrane preparations can be generated that are greater than 50% pure. These efforts led to scores of papers describing biological electron-transfer, and to important international collaborations, including with **Richard Cammack** (Kings College, London, UK), **Fraser Armstrong** (University of Oxford), **Francis Blasco** (CNRS, Marseille), **Gary Cecchini** (UCSF), and **Natalie Strynadka** (UBC). The Cecchini collaboration generated a critical paper describing the role of the heme in *E. coli* succinate dehydrogenase [2007, *Proc. Natl. Acad. Sci. USA* **104**(46), 18007, *cited 35 times*].

2. Solving the Structure of NarGHI (2003). *E. coli* nitrate reductase is a large membrane-bound dimer of heterotrimers of ~500 kDa molecular weight. I applied my expertise in protein overexpression and purification to generate highly pure and crystallizable enzyme preparations which led to a 1.9Å resolution structure, a remarkable achievement in membrane protein structure determination at the time (in collaboration with **Natalie Strynadka**) [2003, *Nature Structural* Biology **10**(9), 681, *cited 297 times*]. This led to a number of additional contributions, including a detailed structural description of quinol binding to the enzyme [2005, *J. Biol. Chem.* **280**(15), 14836, *cited 49 times*].

3. Discovery of the Tat Translocase (1998). This solved the problem of how fully-folded cofactor-containing enzymes can be translocated across membranes, and spawned an entirely new field that is being pursued by many laboratories around the world [1998, *Cell* **93**(1), 93, *cited 276 times*]. I hold two patents on biotechnology applications for the Tat system.

4. Discovery of the YebF Protein Export System (2005). This system is able to secrete mature proteins into *E. coli* growth media and is the subject of two patents, with the technology being marketed by Athena Expression Systems (www.athenaes.com/ACES_yebf_kit.php). Studies on YebF generated two high-impact papers [2005, *Nat. Biotech.* **24**(1), 100, *cited 45 times*; 2012, *Structure* **20**(7), 1154, *cited 6 times*].

5. Determination of the Role of the Pyranopterin Component of the Molybdenum Cofactor (2012). The structural complexity of the molybdenum cofactor led me to challenge the metallocentric view of molybdoenzyme function. By carefully analyzing pyranopterin conformations in available protein structures, I demonstrated the existence of an additional redox state of pyranopterin (the dihydro *versus* tetrahydro form, in collaboration with **Martin Kirk,** University of New Mexico). This work has had a pivotal impact in developing an understanding the remarkable substrate diversity of molybdoenzymes [2012, *Proc. Natl. Acad. USA* **109**(37), 14773, *cited 10 times*].

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Table 1: Abbreviations

Abbreviation	***Definition***
DmsABC	*E. coli* dimethylsulfoxide reductase
DMSOR	Dimethylsulfoxide reductase
E_m	Reduction potential
EPR	Electron paramagnetic resonance
IRTG	International Research Training Group
MCD	Magnetic circular dichroism
Moco	Mononuclear molybdenum cofactor
Mo-enzymes	Molybdoenzymes
Mo-PPT	Molybdo-pyranopterin
Mo-PCD	Molybdo-(pyranopterin cytosine dinucleotide)
Mo-bisPGD	Molybdo-bis(pyranopterin guanine dinucleotide)
MPDRG	CIHR Molecular Biology of Membranes Research Group
NarGHI	*E. coli* respiratory nitrate reductase
PFV	Protein film voltammetry
PPT	Pyranopterin
SUOX	Sulfite oxidase
XAS	X-ray absorption spectroscopy
XDH	Xanthine dehydrogenase

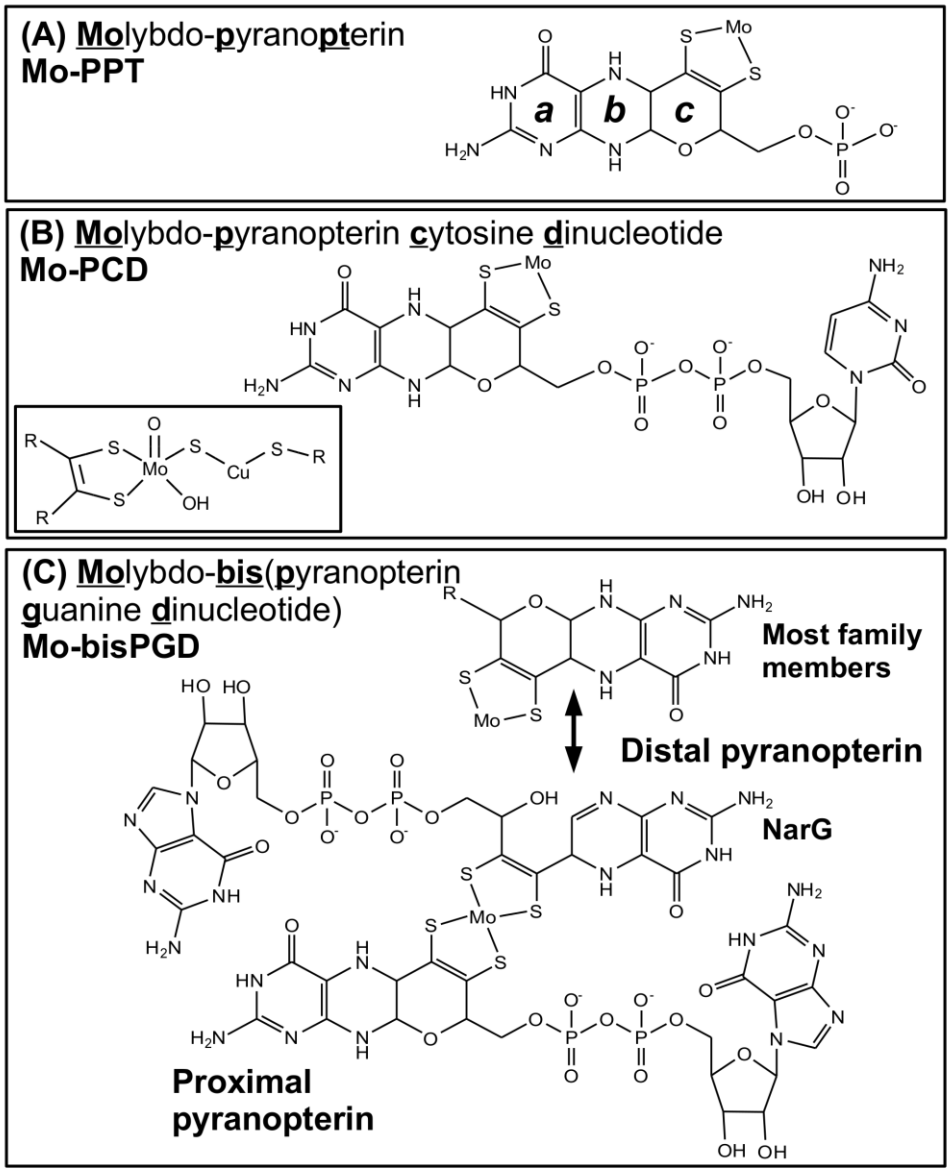

Figure 1: ***Scalable complexity of the molybdenum cofactor.*** (**A**) The Molybdo-pyranopterin cofactor (Mo-PPT) found in eukaryotic molybdoenzymes, and bacterial xanthine dehydrogenases (XDH) and sulfite oxidases (SUOX). The tricyclic pyranopterin comprises (***a***) pyrimidine, (***b***) pyrazine, and (***c***) pyran rings. (**B**) The molybdo-(pyranopterin cytosine dinucleotide) cofactor (Mo-PCD) found in most bacterial XDH-fold enzymes. Inset – a variation found in some CO dehydrogenases. (**C**) The molybdo-bis(pyranopterin guanine dinucleotide) cofactor (Mo-bisPGD) found in the majority of bacterial molybdoenzymes. In two examples, the *E. coli* respiratory nitrate reductase (NarGHI) and the ethylbenzene dehydrogenase (EbdABC) from *Aromatoleum aromaticum*, one of the pyranopterins has a bicyclic structure.

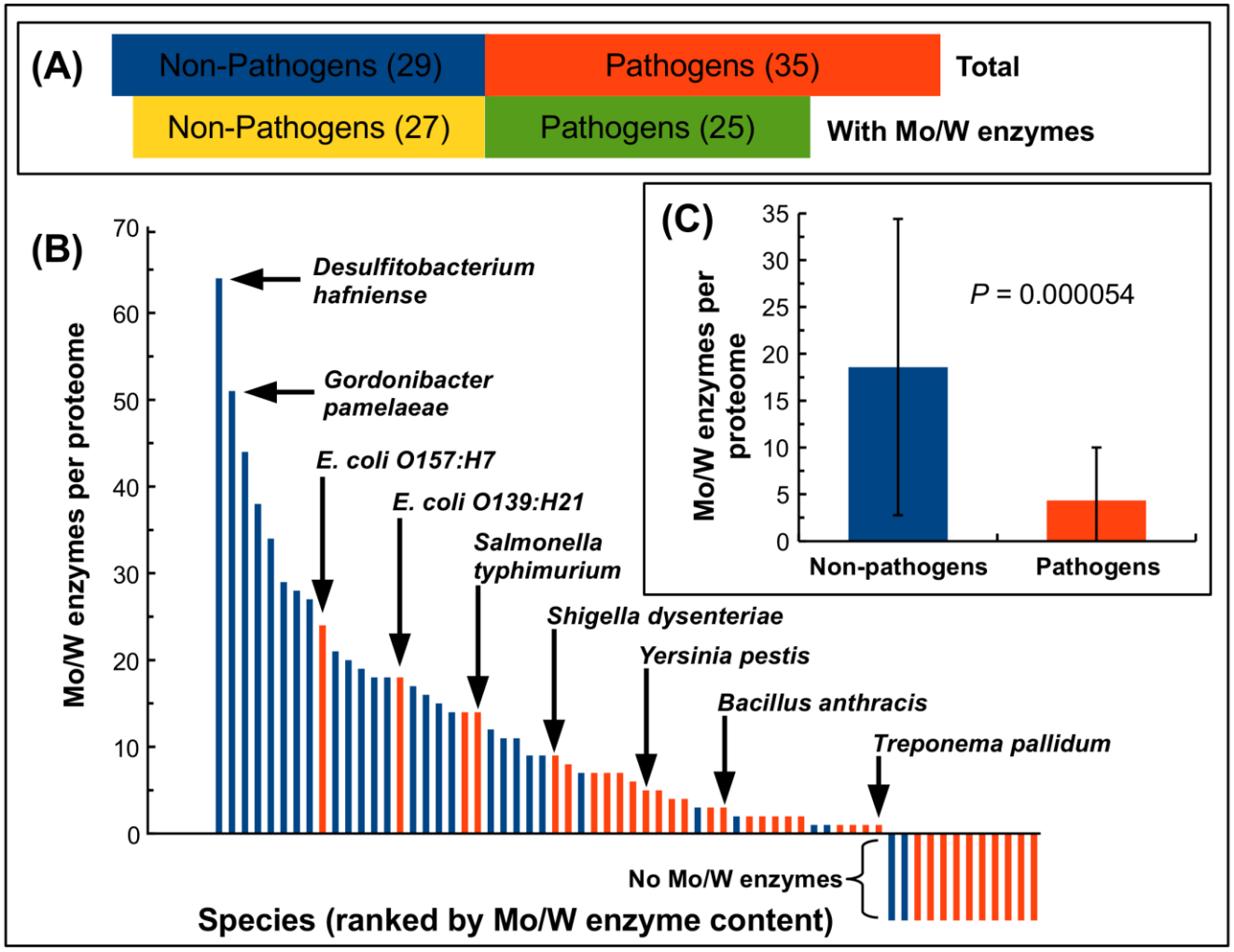

Figure 2: ***Inverse correlation between pathogenicity and the presence of Mo/W-enzymes in selected non-pathogens and pathogens***. Note that in these analyses enzymes containing the mononuclear tungsten cofactor are also included. (**A**) Chart showing that the occurrence of predicted Mo/W-enzymes is biased against strain pathogenicity. Numbers in parentheses indicate either the number of pathogenic or non-pathogenic strains, or the number of strains with ≥1 predicted Mo/W enzyme. Of the 64 predicted proteomes analyzed, 2 and 10 non-pathogens and pathogens lacked Mo/W enzymes, respectively. (**B**) Plot of the number of predicted Mo/W enzymes per proteome versus species ranked by Mo/W-enzyme content. Data bars for non-pathogens are colored blue, and those for pathogens are colored red. Proteomes lacking predicted Mo/W-enzymes are indicated by the presence of an appropriately-colored bar below the abscissa. (**C**) Comparison of the mean values for Mo/W-enzyme content amongst non-pathogens (18.6 ± 15.8, n = 29) and pathogens (4.3 ± 5.6, n = 35). A t-test revealed a P-value of 0.000054 between the non-pathogen and pathogenic datasets.

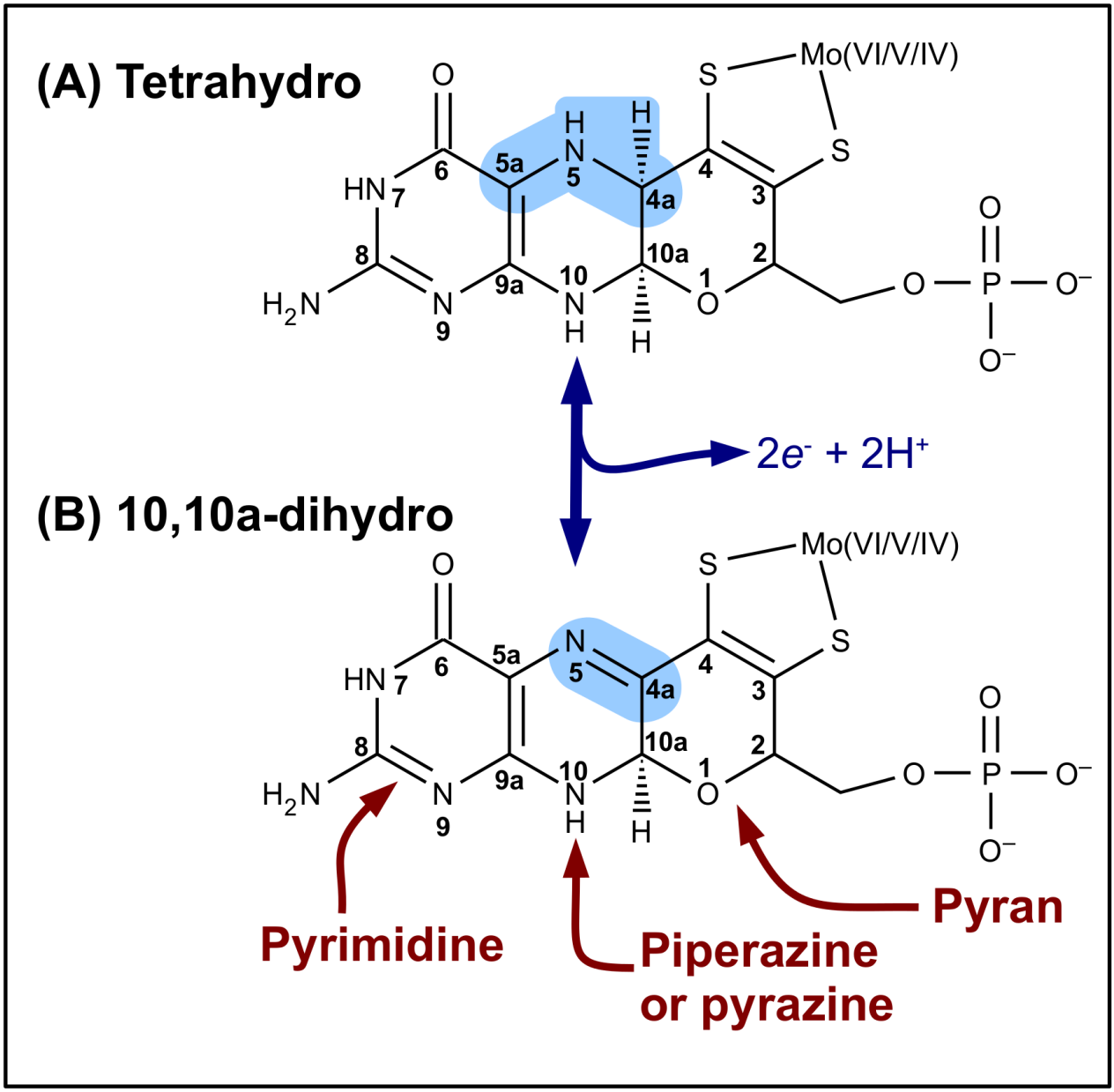

Figure 3: ***Oxidation states of the pyranopterin cofactor***. Interconversion of the tetrahydro (**A**) and 10,10a-dihydro (**B**) forms. The blue shading indicates the atoms affected by the indicated oxidation reaction.

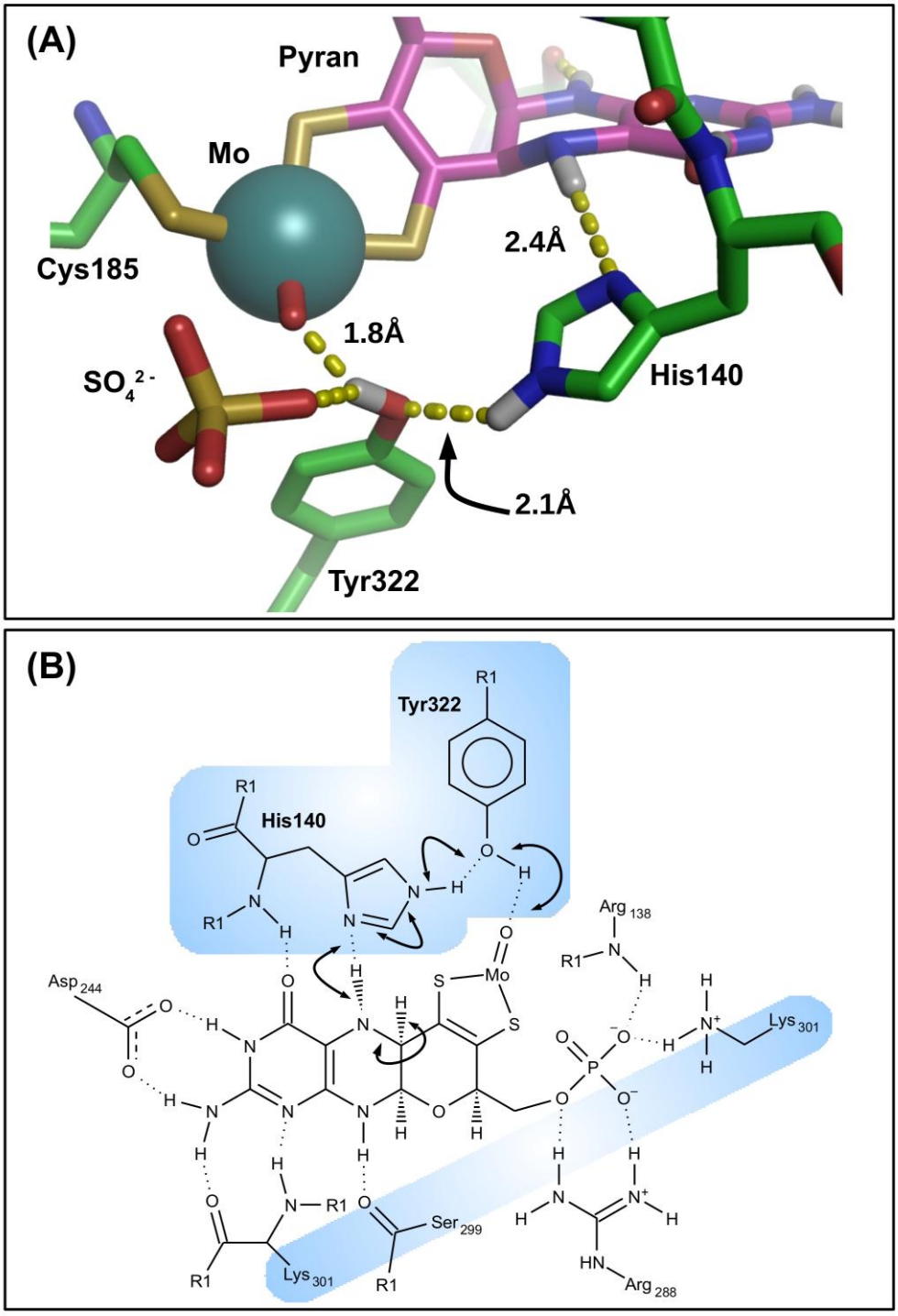

Figure 4: ***SUOX-fold charge-transfer relay*** (**A**) Charge-transfer relay connecting the *N*-5 atom of the pyranopterin to the molybdenum atom in sulfite oxidase subfamily of the SUOX fold enzymes. A conserved His and Tyr are observed connecting the N-5 hydrogen of the piperazine ring to the Mo active site. (**B**) Summary of contact residues and proposed mechanism of charge transfer-mediated redox interconversion.

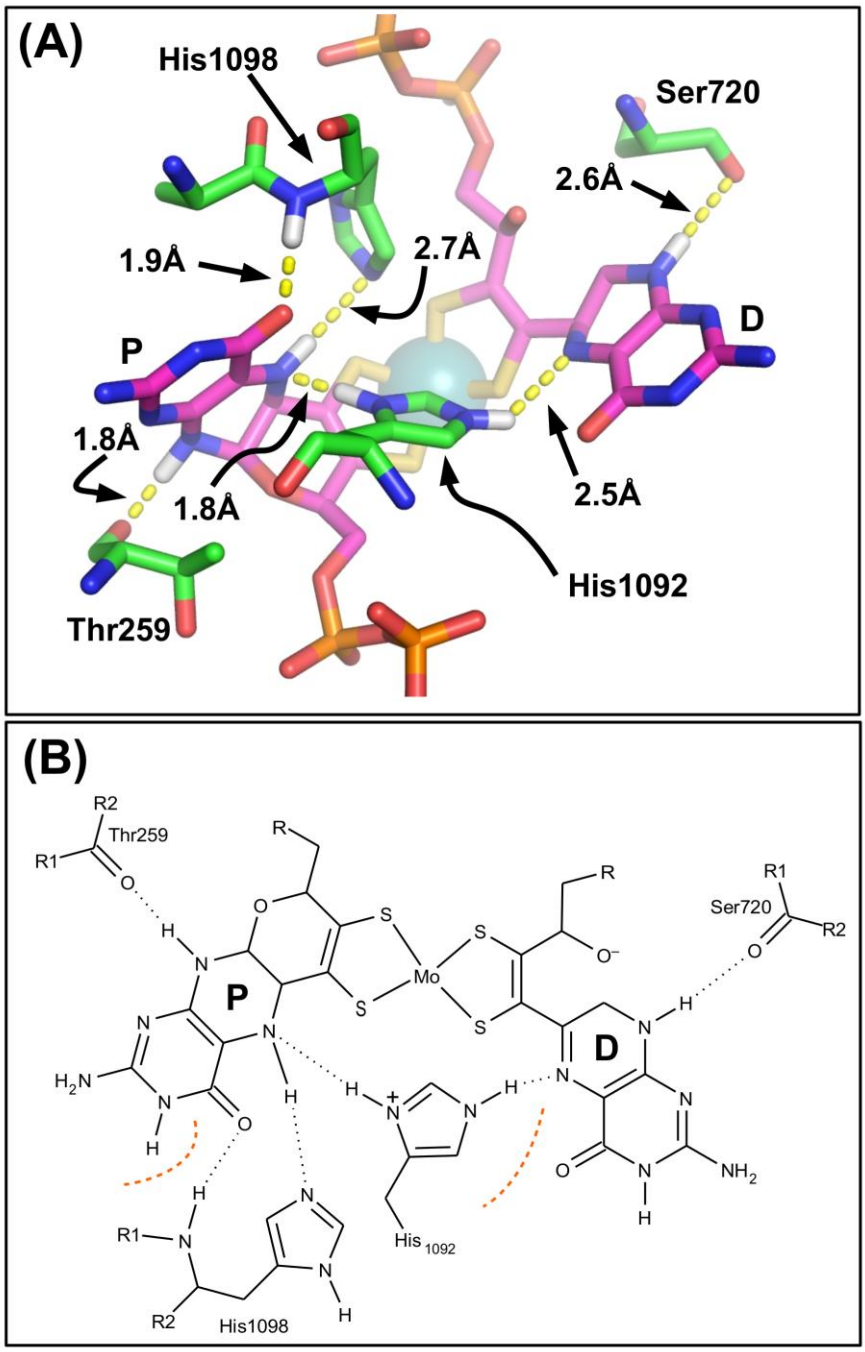

Figure 5: ***Pyranopterin interactions in NarGHI*** (**A**) Residues controlling piperazine ring coordination. A conserved His functions to bridge the two pyranopterins, while another His residue participates in a second interaction with the N-5 atom of one of the pyranopterins (B) Proposed H-bonding network around the piperazine rings of the two pyranopterins. One pyranopterin is shown in its tetrahydro form (**P**), with this redox state stabilized by both the conserved bridging His and the second His residue (**D**).

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