Protein-bound molybdenum cofactor is bioavailable and rescues molybdenum cofactor-deficient *C. elegans*

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Abstract:

The molybdenum cofactor (Moco) is a 520 dalton prosthetic group that is synthesized in a multi-step enzymatic pathway present in most Archaea, Bacteria, and Eukarya. In animals, four oxidases (among them sulfite oxidase) use Moco as a prosthetic group. Moco is essential in animals; humans with mutations in genes that encode Moco-biosynthetic enzymes display lethal neurological and developmental defects. Moco supplementation seems a logical therapy, however the instability of Moco has precluded biochemical and cell biological studies of Moco transport and bioavailability. The nematode *Caenorhabditis elegans* can take up Moco from its bacterial diet and transport it to cells and tissues that express Moco-requiring enzymes, suggesting a system for Moco uptake and distribution. Here we show that protein-bound Moco is the stable, bioavailable species of Moco taken up by *C. elegans* from its diet and is an effective dietary supplement in a *C. elegans* model of Moco deficiency. Diverse purified Moco:protein complexes from bacteria, bread mold, green algae, and dairy cows were able to support the growth of otherwise Moco-deficient *C. elegans* mutants grown on Moco-deficient *E. coli.* We show that these Moco:protein complexes are very stable, suggesting they may provide a strategy for the production and delivery of therapeutically active Moco to treat human Moco deficiency.

36 **Introduction:**

37 The molybdenum cofactor (Moco) is an ancient coenzyme that was present in the last
38 universal common ancestor and that continues to be synthesized in all domains of life (1, 2).
39 Moco is a pterin-based organic prosthetic group that is comprised of a C6-substituted pyrano
40 ring, a terminal phosphate, and a dithiolate group binding to molybdenum (**Fig. 1A**) (3). In
41 humans and other animals, Moco is required for the activity of 4 enzymes: sulfite oxidase,
42 xanthine oxidase, aldehyde oxidase, and mitochondrial amidoxime reducing component (4).
43 There are 2 forms of eukaryotic Moco, the sulfite oxidase form and the xanthine oxidase form
44 (**Fig. 1A).** These Moco species differ in the third Mo-S ligand which is provided either by an
45 enzyme-derived cysteine residue (sulfite oxidase form) or an inorganic sulfur (xanthine oxidase
46 form) (4). The xanthine oxidase form of Moco is synthesized from the sulfite oxidase form via
47 the enzyme Moco sulfurase (**Fig. 1A**) (5).

48 Both forms of Moco are synthesized by a highly conserved biosynthetic pathway (**Fig.**
49 **1A**) (6). The genes necessary for Moco biosynthesis were first elucidated by genetic studies of
50 chlorate resistance in bacteria (7). The importance of Moco biosynthesis to human health is
51 highlighted by Moco deficiency (MoCD), a rare inborn error of metabolism. MoCD is caused by
52 loss-of-function mutations in genes encoding any of the human Moco-biosynthetic enzymes and
53 results in severe neurological dysfunction and neonatal lethality (8, 9). MoCD patients with
54 mutations in MOCS1 (orthologous to bacterial *moaA* and *moaC*) can be treated with cyclic
55 pyranopterin monophosphate (cPMP), a stable intermediate in Moco biosynthesis immediately
56 downstream of MOCS1 (10). However, cPMP treatment is not effective for patients with
57 mutations in any of the downstream Moco-biosynthetic enzymes. Purification and delivery of
58 mature Moco would be an ideal therapeutic strategy for treating all forms of MoCD, however
59 free Moco is too unstable and oxygen-sensitive to be purified and therapeutically administered.
60 Furthermore, it is unclear whether mature Moco can cross cellular membranes.

61 Genetic evidence demonstrates that the nematode *C. elegans* retrieves Moco as well as
62 cPMP from its bacterial diet, *E. coli* (11). However, nothing was known about the biochemical
63 mechanism of Moco transfer between these 2 highly divergent organisms. (11). Here we
64 propose that Moco bound to protein is the stable and bioavailable Moco species that is being
65 harvested by *C. elegans.* We demonstrate that supplementation of purified protein-bound Moco
66 rescues the lethality of Moco-deficient *C. elegans* feeding on Moco-deficient *E. coli*. We show
67 that Moco bound to diverse Moco-containing proteins originating from bacteria, algae, fungi, and
68 mammals, is bioavailable to *C. elegans,* and that this supplementation does not require Moco
69 biosynthetic enzymes in *C. elegans* or its bacterial diet. This work suggests future mammalian

70 therapeutic studies of supplemental protein-bound Moco and highlights the existence of a
71 pathway for Moco transport and harvest.
72
73 **Results and Discussion:**
74 *C. elegans* acquires Moco from dietary *E. coli*:
75 Due to its instability, Moco has long been thought to be synthesized and utilized cell
76 autonomously with no evidence for transport between cells, tissues, and organisms. So far only
77 *C. elegans* has been described to have 2 pathways by which it can obtain Moco: endogenous
78 Moco biosynthesis from GTP or dietary uptake of Moco, which demands other genetic pathways
79 of intestinal uptake from the bacterial diet and transport of Moco to client tissues (11). Moco-
80 biosynthetic enzymes are conserved in all domains of life; in *C. elegans* these enzymes are
81 encoded by *moc-1, moc-2, moc-3, moc-4,* and *moc-5* that mediate sequential steps in Moco
82 biosynthesis **(Fig. 1A)**. Using mutations in the *C. elegans moc* genes (i.e. the *moc-1(ok366)* null
83 mutation) Moco biosynthesis can be interrupted in all cells. In the laboratory, *C. elegans* feed on
84 a monoculture of *E. coli*. Thus, we can also use mutations in any of the genes of the *E. coli*
85 Moco-biosynthetic pathway to eliminate dietary Moco (i.e. the Δ *moaA* null mutation). Either
86 endogenous Moco synthesis in *C. elegans* or Moco produced by the diet *E. coli* and then
87 consumed by *C. elegans* can support growth, development, and reproduction of *C. elegans*.
88 However, when *C. elegans* cannot synthesize their own Moco and cannot obtain Moco from
89 their diet, they arrest in larval development and ultimately die due to inactivity of sulfite oxidase,
90 the key Moco-utilizing enzyme in animals (11).
91 To test how much wild-type, Moco-producing bacteria was required to support growth
92 and development of *C. elegans* defective in Moco biosynthesis, we mixed wild-type and Δ *moaA*
93 mutant *E. coli* at various ratios and tested for the ability of these mixtures to support the viability
94 of *moc-1* mutant *C. elegans.* We speculated that if Moco functions like a vitamin, only trace
95 amounts of wild-type, Moco-producing bacteria would be needed to support *moc-1* mutant
96 viability. However, we found that about 30% of the bacterial diet needed to be wild type (Moco
97 producing) to support growth and development of *moc-1* mutant animals (**Fig. 1B)**.
98
99 Diverse protein-bound Moco is taken up and utilized by *C. elegans*:
100 We hypothesized that *C. elegans* harvest bacterial Moco that is stably bound within the
101 *E. coli* Moco-utilizing enzymes. *E. coli* YiiM (*Ec*YiiM) is one such Moco-utilizing enzyme and
102 mediates the reduction of N-hydroxylated substrates (12, 13). To test whether Moco bound to
103 *Ec*YiiM can be absorbed by *C. elegans*, we purified recombinant *Ec*YiiM protein from *E. coli* and

104 used it to supplement the diet of Moco-biosynthetic mutant *C. elegans* feeding on Moco-
105 deficient *E. coli,* growth conditions that would otherwise result in 100% larval arrest and death.
106 Consistent with the model that *C. elegans* utilizes Moco from *E. coli* Moco-utilizing enzymes,
107 *moc-1* mutant animals grown on Moco-deficient *E. coli* grew and developed well when their diet
108 was supplemented with *Ec*YiiM-bound Moco. (**Fig. 2A,B**). Thus, *Ec*YiiM-bound Moco is
109 bioavailable and can support the viability of otherwise Moco-deficient *C. elegans*.
110 To test if the ability of *C. elegans* to harvest Moco from protein was more general to other
111 Moco-binding proteins, we recombinantly expressed and purified two additional Moco-binding
112 proteins in *E. coli*; nitrate reductase from the red bread mold *Neurospora crassa* (*Nc*NR) and
113 Moco-carrier protein from the green algae *Volvox carteri* (*Vc*MCP) (14-16). We also utilized the
114 commercially available Moco-using enzyme xanthine oxidase (*Bt*XO) purified from bovine milk
115 (17). Each Moco-binding protein was supplemented to *moc-1* mutant *C. elegans* fed Δ*moaA*
116 mutant *E. coli*. Similar to *Ec*YiiM, supplementation with Moco bound to either *Vc*MCP or *Nc*NR
117 supported the growth of *moc-1* mutant *C. elegans* in the absence of any other dietary Moco
118 (**Fig. 2)**. To a lesser extent, *Bt*XO supplementation also supported the growth of *moc-1* mutant
119 animals cultured on Moco-deficient *E. coli* (**Fig. 2A,B)**. A possible explanation for the reduced
120 efficacy of supplemental *Bt*XO compared to *Ec*YiiM, *Vc*MCP, or *Nc*NR might be the form of
121 Moco that is bound by these proteins. *Bt*XO binds the xanthine oxidase form of Moco while
122 *Ec*YiiM, *Vc*MCP, and *Nc*NR bind the sulfite oxidase form of Moco (**Fig. 1A**) (12, 15, 16, 18). In
123 *C. elegans* and other animals sulfite oxidase (SUOX-1) is the key Moco-requiring enzyme
124 necessary for viability; *suox-1* null mutant animals arrest development similar to Moco-deficient
125 animals (11). It is reasonable that protein supplementation with the sulfite oxidase form of Moco
126 can supply the appropriate Moco to support *C. elegans* SUOX-1 activity compared to a xanthine
127 oxidase form of Moco. Alternatively, supplementation with the sulfite oxidase form of Moco may
128 result in the partial conversion, via Moco sulfurase (encoded by *C. elegans mocs-1*), of that
129 supplemental Moco into the xanthine oxidase form (**Fig. 1A**). Thus, by providing the sulfite
130 oxidase form of Moco we may be providing both forms of eukaryotic Moco making it a more
131 effective treatment for complete Moco deficiency in *C. elegans*. Supplementation with the
132 xanthine oxidase form of Moco would likely not result in synthesis of the sulfite oxidase form of
133 Moco as there is no known enzyme that desulfurates the xanthine oxidase form of Moco.
134 To further demonstrate that the growth of *C. elegans moc-1* mutant animals was
135 conferred by supplementation of the Moco prosthetic group and not by the supplemental purified
136 proteins, we purified apo-*Vc*MCP from bacteria unable to synthesize Moco. Supplemental apo-
137 *Vc*MCP, did not support the growth of *moc-1* mutant *C. elegans* fed Δ*moaA* mutant *E. coli* (**Fig.**

138 **2A,C,D)**. Taken together these data demonstrate that the animal *C. elegans* is able to acquire
139 and harvest the Moco prosthetic group when it is provided as a dietary supplement in complex
140 with Moco-binding proteins. These proteins have diverse structures and functions and originate
141 from both prokaryotes and eukaryotes. As such, the acquisition of protein-bound Moco by *C.*
142 *elegans* is not specific to certain Moco-binding proteins and may reflect a general strategy for
143 acquisition of functional Moco from the animals' diet or microbiome. Furthermore, as Moco
144 biosynthesis and utilization are ancient processes conserved in all domains of life, we believe it
145 is unlikely that a novel biochemical pathway for Moco transfer across cell membranes has
146 evolved exclusively in the nematode *C. elegans.* However, it remains to be tested whether
147 protein-bound Moco can permeate the cells of other organisms. The remaining experiments
148 were all performed with supplemental Moco bound to *Vc*MCP due to its well-characterized role
149 in Moco binding and our established protocols for its production (14, 16).
150 One model for the rescue of *C. elegans* Moco deficiency is that supplemental protein-
151 bound Moco is directly ingested by *C. elegans.* Alternatively, the protein-bound Moco may first
152 be taken up by *E. coli* which may process the Moco to then be ingested by *C. elegans.* To
153 distinguish between these models, we grew Δ*moaA* mutant *E. coli* in lysogeny broth (LB)
154 supplemented with Moco bound to *Vc*MCP. This Δ*moaA E. coli* was then separated from the
155 culture medium by centrifugation, washed extensively, and fed to *moc-1* mutant *C. elegans*
156 ("Diet B", **Fig. 3)**. Although cultured with Moco bound to *Vc*MCP, the washed Δ*moaA E. coli* in
157 Diet B did not support growth of *moc-1* mutant animals. Importantly, the supernatant medium
158 from the same culture supported the growth of *moc-1* mutant *C. elegans* grown on a lawn of Δ
159 *moaA E. coli* grown separately in LB alone ("Diet A", **Fig. 3B)**. Together, these data suggest that
160 supplemental protein-bound Moco does not pass through a bacterial intermediate before being
161 acquired by *C. elegans* (**Fig. 3B)**.
162
163 <u>Moco bound to protein is stable:</u>
164 The instability and oxygen sensitivity of Moco has limited cell biological studies of Moco
165 transport and precluded it from therapeutic consideration (19). The *Vc*MCP-bound Moco used in
166 "Diet A" (**Fig. 3)** was incubated at 37°C overnight, and still retained its activity and bioavailability,
167 suggesting remarkable stability. To biochemically demonstrate the stability of Moco bound to
168 protein, we measured the ability of mature Moco to stay in complex with *Vc*MCP, *Ec*YiiM, *Nc*NR,
169 and *Bt*XO over time (**Fig. 4**). Free Moco is highly unstable, however it can be oxidized to 'Form
170 A', a stable and fluorescent Moco-derivative that is quantifiable via HPLC (20, 21). Using

171 measurements of Form A and protein concentration, we first determined the initial Moco
172 occupancy of purified *Vc*MCP (22%) as well as *Ec*YiiM (4%), *Nc*NR (50%), and *BtXO* (50%)
173 (**Fig. 4A,B**). We then assessed the stability of each purified Moco:protein complex by
174 determining Moco retention over time at different temperatures (**Fig. 4C-F)**. All 4 Moco:protein
175 complexes were remarkably stable, showing no significant protein degradation and retaining
176 between 43 and 83% of their original Moco content after 96 hours of incubation at ambient
177 temperature (**Fig. 4C-F**). This stability is surprising and suggests purification of protein-bound
178 Moco as a new strategy for the production and delivery of therapeutically active Moco to treat
179 MoCD.
180
181 <u>Bioavailability of recombinant protein-bound Moco does not depend on known Moco-</u>
182 <u>biosynthetic enzymes in *E. coli* or *C. elegans*:</u>
183 We tested if the Moco-biosynthetic enzymes are necessary for the harvesting or transport
184 of supplemental protein-bound Moco using mutants in the dietary *E. coli*. We tested *moc-1*
185 mutant *C. elegans* growth on wild-type bacteria, or mutant bacteria lacking the genes necessary
186 for Moco biosynthesis (*moaA, moaC, moaD, moaE, moeB, mog, moeA, modA, modC*, or *ydaV*).
187 *moc-1* mutant animals were grown on mutant *E. coli* with and without supplemental Moco bound
188 to *Vc*MCP. *moc-1* mutant *C. elegans* grew well on wild-type *E. coli* but displayed larval arrest on
189 all 10 *E. coli* mutants defective in Moco biosynthesis (**Fig. S1)**. Supplemental Moco bound to
190 *Vc*MCP supported growth and development of *moc-1* mutant *C. elegans* on all 10 of the Moco-
191 biosynthetic mutant *E. coli* demonstrating that none of these *E. coli* genes were necessary for
192 bioavailability of supplemental protein-bound Moco (**Fig. S1)**.
193 Alternatively, we speculated that the Moco-biosynthetic machinery of *C. elegans* might
194 play a role in the bioavailability of supplemental protein-bound Moco. To test this, we used
195 established *C. elegans* mutants in the Moco-biosynthetic pathway (*moc-5, moc-4, moc-3, moc-*
196 *2,* and *moc-1*, **Fig. 1A**). Each of these *C. elegans* mutants was cultured on wild-type *E. coli*, Δ
197 *moaA E. coli,* or Δ*moaA E. coli* supplemented with Moco bound to *Vc*MCP. All of the *moc*
198 mutant animals grew well on wild-type bacteria that produce Moco and arrested growth on Δ
199 *moaA E. coli* that lacks Moco biosynthesis (**Fig. S2A-E**). Each *C. elegans moc*-mutant displayed
200 dramatically improved growth on Δ*moaA E. coli* when their diet was supplemented with Moco
201 bound to *Vc*MCP (**Fig. S2A-E**). These results demonstrate that *moc-5, moc-4, moc-3, moc-2,*
202 and *moc-1* are not required for the bioavailability of supplemental protein-bound Moco. Thus,

203 the machinery that facilitates Moco transport is distinct from the canonical Moco biosynthetic
204 pathway.
205
206 Supplemental protein-bound Moco supports the activity of *C. elegans* SUOX-1:
207 The lethality associated with Moco deficiency in *C. elegans* and humans is due to
208 inactivity of sulfite oxidase (SUOX-1), a mitochondrial Moco-requiring enzyme that oxidizes the
209 lethal toxin sulfite to sulfate. Like Moco-biosynthesis, sulfite oxidase is essential in both *C.*
210 *elegans* and humans (11, 22). Thus, to rescue development of Moco-deficient *C. elegans,*
211 supplemental protein-bound Moco must be incorporated into and support the activity of *C.*
212 *elegans* SUOX-1. To demonstrate this, we utilized the hypomorphic *suox-1* allele *gk738847*
213 (D391N) (23). Aspartic acid 391 of sulfite oxidase is highly conserved and is present in *C.*
214 *elegans, Drosophila melanogaster, Danio rerio, Mus musculus,* and *Homo sapiens*. The SUOX-
215 1 D391N amino acid substitution causes partial SUOX-1 loss-of-function that is enhanced when
216 dietary Moco is absent. Growing *suox-1(gk738847)* mutant *C. elegans* on Moco-deficient *E. coli*
217 causes a severe developmental delay compared to its growth on wild-type Moco-producing *E*
218 *coli* (**Fig. S2F**) (11). Importantly, *suox-1(gk738847)* mutant animals are wild type for their
219 endogenous Moco biosynthetic pathway and are able to synthesize Moco *de novo*. This result
220 shows that *C. elegans* depends on both endogenous Moco biosynthesis as well as dietary
221 sources of Moco to fully support the activity of SUOX-1.
222 We hypothesized that supplemental Moco bound by *Vc*MCP would improve the viability
223 of *suox-1(gk738847)* animals grown on Δ*moaA E. coli.* To test this, we cultured *suox-*
224 *1(gk738847)* mutant animals on wild-type *E. coli*, Δ*moaA E. coli,* and Δ*moaA E. coli*
225 supplemented with Moco bound to *Vc*MCP. Consistent with our rescue of *C. elegans* Moco
226 deficiency, supplemental protein-bound Moco improved the growth of *suox-1(gk738847)*
227 animals grown on Moco-deficient *E. coli* (**Fig. S2F)**. These results suggest that exogenous
228 protein-bound Moco is absorbed, harvested, distributed to requisite cells and tissues, and re-
229 inserted into the *C. elegans* SUOX-1 enzyme.
230 Our data demonstrate the ability of an essential protein-packaged prosthetic group to
231 cross cell membranes. This transfer naturally occurs between multiple organisms (i.e. from *E.*
232 *coli* to *C. elegans*) and among the cells and tissues of a single organism; Moco absorbed in the
233 intestine of *C. elegans* must cross multiple cell membranes to reach all of the Moco-utilizing
234 cells and tissues. Because Moco biosynthesis is as ancient as the last universal common
235 ancestor, it is likely that the not yet discovered Moco-transport pathway may be general to all
236 animals, including humans. Furthermore, roughly 70% of bacterial genomes encode Moco-

237 biosynthetic enzymes making the intestinal microbiome a potential reservoir for this cofactor (2).
238 Similarly, the human diet might also be a source of exogenous protein-bound Moco as most
239 plants and animals synthesize and utilize Moco. Our results with the nematode *C. elegans* may
240 stimulate future exploration of the therapeutic potential of protein-bound Moco from dietary,
241 microbiome, or recombinant sources.
242
243 **Materials and Methods:**
244 General methods and strains:
245 *C. elegans* strains were cultured at 20°C on nematode growth medium (NGM) seeded
246 with wild-type *Escherichia coli* unless otherwise noted (24). The wild-type strain of *C. elegans*
247 was Bristol N2. For each linkage group, the *C. elegans* mutant strains used in this work and
248 their associated genotypes are listed. LGI: GR2253 *moc-4(ok2571).* LGIV: MH3266 *moc-*
249 *3(ku300).* LGV: GR2255 *moc-2(mg595).* LGX: GR2254 *moc-1(ok366),* GR2256 *moc-5(mg589),*
250 and GR2269 *suox-1(gk738847).*
251 *E. coli* strains were cultured using standard methods. The wild-type strain of *E. coli* was
252 BW25113, the parental strain of the Keio *E. coli* knockout collection (25). The *E. coli* mutants
253 used in this work were JW0764 (Δ *moaA::*Kan^r), KJW1 (Δ *mog*), KJW2 (Δ *moaA*), KJW3 (Δ
254 *moaC*), KJW4(Δ *moaD*), KJW5(Δ *moaE*), KJW6(Δ *moeB*), KJW7(Δ *moeA*), KJW8(Δ *modA*),
255 KJW9(Δ *modC*), and KJW10(Δ *ydaV*). KJW1-KJW10 are bacterial strains derived from the Keio
256 *E. coli* knockout collection and have been modified using established methods to remove the
257 kanamycin resistance cassette from each locus of interest (11, 25). Strains KJW1-KJW10 were
258 only used to produce the data in Figure S1. JW0764 was used in all other experiments with Δ
259 *moaA E. coli.*
260 *C. elegans* growth assays:
261 *C. elegans* were synchronized at the first stage of larval development (L1). L1 animals
262 were then cultured on NGM seeded with wild-type or mutant *E. coli.* For some experiments,
263 mutant *E. coli* was supplemented with various forms and amounts of protein-bound Moco (see
264 Dietary supplementation with protein-bound Moco). *C. elegans* animals were then allowed to
265 grow and develop for 48 or 72 hours (specified in Figure Legends) at 20°C. For each
266 experiment, the sample size (n) is individual animals measured and is reported in the Figures
267 and Figure Legends.
268 For all assays, live animals were imaged using an Axio Zoom.V16 microscope (Zeiss)
269 equipped with an ORCA-Flash4.0 digital camera (Hamamatsu). Images were captured using

270 ZEN software (Zeiss) and processed utilizing ImageJ (NIH). Animal length was measured from
271 the tip of the head to the end of the tail. The median and upper and lower quartiles were
272 calculated using GraphPad Prism software.

273 Purification and characterization of Moco-binding proteins:

274 Moco-binding proteins were expressed and purified using standard methods (16). The
275 full-length *yiiM* coding sequence was amplified from *Escherichia coli* DH5α (*Ec*YiiM) and the full-
276 length Moco carrier protein coding sequence from *Volvox carteri* (*Vc*MCP) was synthesized and
277 codon optimized for *E. coli* (16). The coding sequence for *Neurospora crassa* nitrate reductase
278 (*Nc*NR) was shortened to include only the Moco-binding and dimerization region (amino acids
279 113-592). The coding sequences for *Ec*YiiM, *Vc*MCP, and *Nc*NR were inserted into the pONE-
280 CP plasmid, producing proteins fused to a C-terminal Streptavidin tag. Streptavidin-tagged
281 proteins were expressed using the *E. coli* strain TP1000 which accumulates the eukaryotic form
282 of Moco due to a deletion in the Mob operon (26). As a negative control, *Vc*MCP was also
283 purified from the *E. coli* strain RK5204 which is unable to produce Moco due to a mutation in
284 *moaE* (27). Bovine xanthine oxidase (*Bt*XO) was purchased from Sigma-Aldrich (X1875, batch
285 SLCB1289).

286 Protein concentrations were determined using absorption at 280 nm and the Pierce BCA
287 Protein-Assay (Thermo Scientific). Absorption was measured using a Multiskan GO Microplate
288 Spectrophotometer (Thermo Scientific). Quantification of Moco content of the proteins was
289 conducted using HPLC-based measurements of Form A, a stable and fluorescent Moco-
290 oxidation product (21). Stability of protein-bound Moco was assessed by incubating the
291 Moco:protein complexes at various temperatures (4°C, 22°C, and 37°C) for 96 hours. In 24-hour
292 intervals, protein samples were centrifuged at 4°C to remove precipitated protein and protein
293 concentration and Moco content were determined as described above.

294 Dietary supplementation with protein-bound Moco:

295 Similar to standard *C. elegans* growth conditions, experiments with supplemental protein-
296 bound Moco were performed on petri dishes filled with approximately 10ml of solidified NGM
297 agar (24). To maximize exposure to the supplemental protein-bound Moco, we resuspended
298 10X concentrated mutant *E. coli* with either 50μl of M9 minimal buffer (control) or 50 microliters
299 of protein-bound Moco in M9. These bacterial resuspensions (with and without protein-bound
300 Moco) were then seeded onto individual petri dishes with NGM and allowed to dry leaving a
301 small concentrated lawn of *E. coli* roughly 1-2 centimeters in diameter. After drying,
302 synchronized L1 *C. elegans* were seeded directly on the lawn of *E. coli* and we proceeded with
303 *C. elegans* growth assays. To maintain consistency among the experiments with supplemental

304 protein-bound Moco, dietary *E. coli* was grown, supplemented with protein-bound Moco, and
305 seeded fresh the same day the *C. elegans* growth assay was to begin.
306 We utilize 4 different sources of protein-bound to Moco to supplement *C. elegans* diets:
307 *Escherichia coli* YiiM (*Ec*YiiM), Neurospora *crassa* nitrate reductase (*Nc*NR), *Volvox carteri*
308 Moco carrier protein (*Vc*MCP), and bovine xanthine oxidase (*Bt*XO). The amount of Moco in
309 every protein preparation was determined experimentally (see Purification and characterization
310 of Moco-bound proteins). The independent variable in our experiments with supplemental
311 protein-bound Moco is the total amount of Moco that is being used to resuspend the dietary *E.*
312 *coli*. Because we do not know the extent to which the protein-bound Moco diffuses throughout
313 the NGM agar, we are limited in our ability to estimate the concentration of protein-bound Moco
314 to which *C. elegans* are exposed. Thus, we report the total amount of Moco used to supplement
315 the *C. elegans* diet and assume equal protein diffusion throughout our 48-72 hour growth
316 experiments.
317 <u>Culturing *E. coli* with Moco bound to *Vc*MCP:</u>
318 Δ*moaA* mutant *E. coli* (JW0764) was cultured in 500μl LB supplemented with 39
319 nanomoles of Moco bound to *Vc*MCP. Cells were cultured overnight at 37°C rotating at 1,400
320 rotations per minute. This overnight culture was then concentrated and the supernatant was
321 removed for use in "Diet A". The concentrated cells were washed repeatedly with M9 minimal
322 buffer, concentrated 10X, and seeded onto NGM agar plates. This diet is referred to as "Diet B"
323 in Figure 3.
324 The supernatant from the overnight culture (see above) was then filtered through a
325 0.20μm filter (Corning) to remove any remaining bacterial cells. This filtered 'spent media' was
326 then used to resuspend a separate concentrated culture of Δ*moaA* mutant *E. coli* (grown only
327 in LB). These resuspended bacterial cells were then seeded onto NGM agar plates. This diet is
328 referred to as "Diet A" in Figure 3.
329
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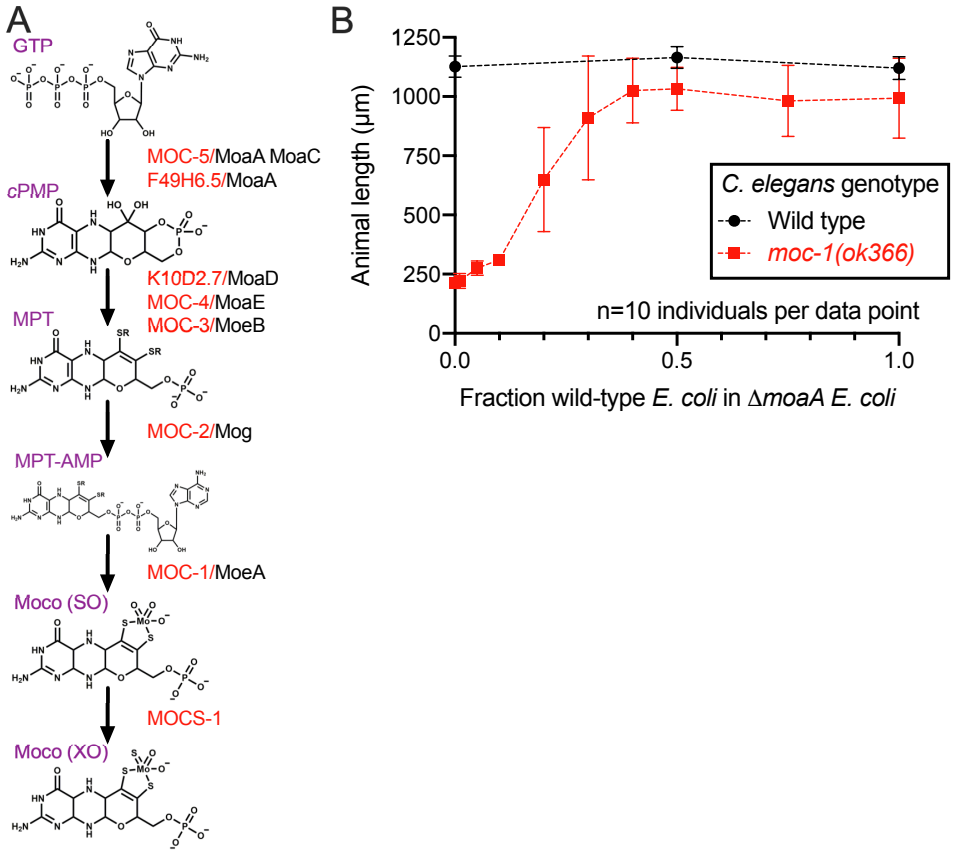
410 **Figure Legends:**



411

412 **Figure 1:** *C. elegans* acquires Moco from dietary *E. coli.*

413 (A) Based on clear protein sequence homologies, the *C. elegans* Moco biosynthesis pathway
414 (red) and orthologous enzymes in *E. coli* (black) are displayed. Moco and its biosynthetic
415 intermediates are displayed (purple): GTP is guanosine triphosphate, cPMP is cyclic
416 pyranopterin monophosphate, MPT is molybdopterin, MPT-AMP is MPT-adenine
417 monophosphate, Moco (SO) is the sulfite oxidase form of the molybdenum cofactor, and Moco
418 (XO) is the xanthine oxidase form of the molybdenum cofactor. *C. elegans* Moco sulfurase
419 (MOCS-1) has no clear homolog in *E. coli,* although *xdhC* is the likely functional analog (28). (B)
420 Wild-type and *moc-1(ok366) C. elegans* were synchronized at the L1 stage and cultured on
421 mixtures of wild-type *E. coli* (synthesizes Moco) and Δ*moaA E. coli* (cannot synthesize Moco) for
422 72 hours. When the bacterial diet was all wild-type *E. coli,* both wild-type and *moc-1(ok366) C.*
423 *elegans* grew and developed well. When the bacterial diet was all Δ*moaA E. coli,* wild-type
424 animals grew well while *moc-1(ok366)* animals arrested growth early in larval development. The
425 Y axis shows animal length (µm), where 1,000µm roughly corresponds with fertile adulthood
426 and 250µm roughly corresponds to the L1 stage. Average and standard deviation are displayed
427 for each condition analyzed. Sample size (n) was 10 individual animals assayed for each
428 condition.

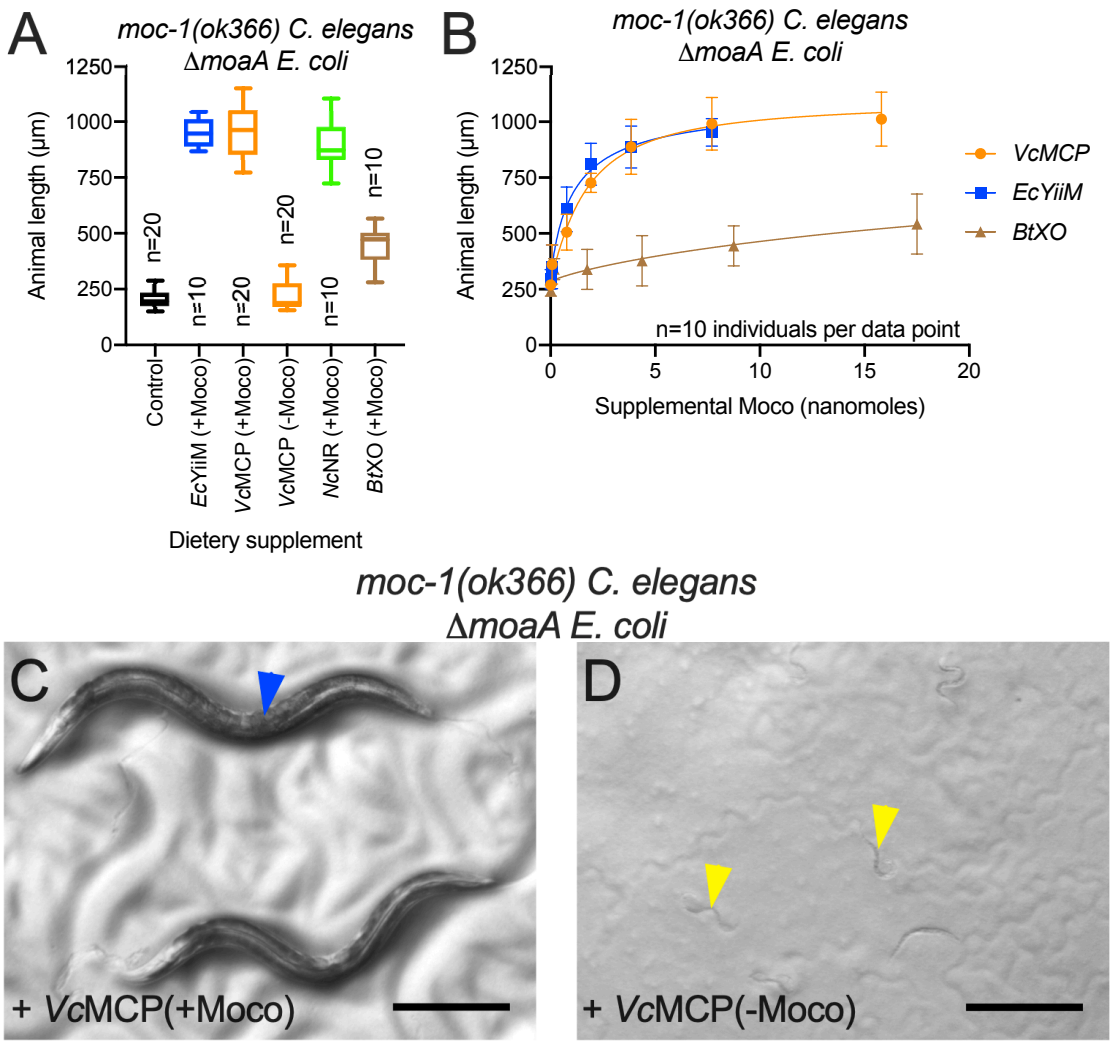


Figure 2: *C. elegans* uses Moco from diverse Moco-containing proteins. (A) *moc-1(ok366)* mutant *C. elegans* were synchronized at the L1 stage and cultured for 72 hours on Δ*moaA E. coli* supplemented with Moco bound to *Escherichia coli* YiiM (*Ec*YiiM), *Volvox carteri* Moco carrier protein (*Vc*MCP), *Neurospora crassa* nitrate reductase (*Nc*NR), or bovine xanthine oxidase (*Bt*XO), or equivalent amounts of *Vc*MCP purified from bacteria that cannot synthesize Moco (-Moco). *Ec*YiiM, *Vc*MCP, and *Nc*NR (+Moco) each contained 7.7 nanomoles of Moco while *Bt*XO (+Moco) contained 8.8 nanomoles of Moco. The Y axis is length (μm), where 1,000μm roughly corresponds with fertile adulthood and 250μm roughly corresponds to the L1 stage. Box plots display the median, upper, and lower quartiles while whiskers indicate minimum and maximum data points. Sample size (n) is displayed for each experiment. (B) *moc-1(ok366)* mutant *C. elegans* were synchronized at the L1 stage and cultured on Δ*moaA E. coli* supplemented with variable amounts of Moco bound to *Ec*YiiM (0.0077, 0.077, 0.77, 1.93, 3.85, or 7.7 nanomoles of Moco, blue), *Vc*MCP, (0.0077, 0.077, 0.77, 1.93, 3.85, 7.7, or 15.8 nanomoles of Moco, orange) or *Bt*XO (0.018, 0.18, 1.8, 4.38, 8.75, or

17.5 nanomoles of Moco, brown). For each experiment, animals were allowed to develop for 72 hours and animal lengths were determined. Mean and standard deviation are displayed for each data point. Sample size (n) was 10 individuals assayed for each data point. (C,D) Representative images of *moc-1(ok366) C. elegans* cultured for 72 hours on Δ*moaA E. coli* supplemented with (C) 7.7 nanomoles of Moco bound to *Vc*MCP or (D) equivalent amounts of apo-*Vc*MCP (-Moco). Blue arrowhead indicates a gravid fertile adult with embryos in its uterus, while yellow arrowheads denote animals arrested early in larval development. Scale bar is 250μm.

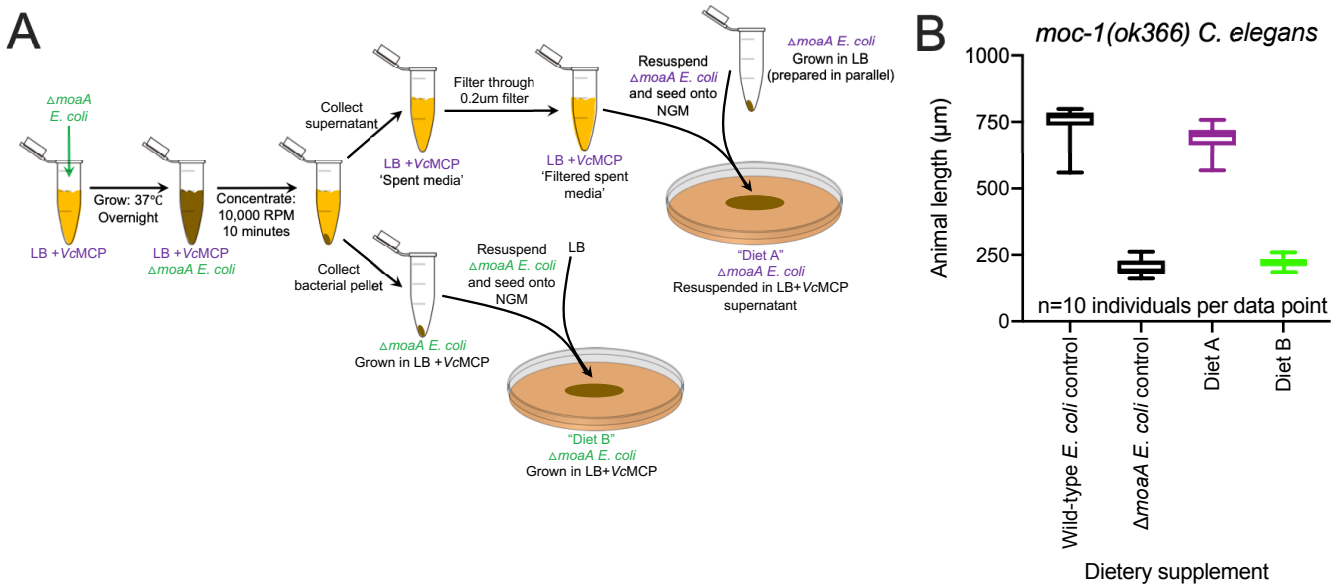


Figure 3: Protein-bound Moco is directly ingested by *C. elegans*
(A) Cartoon of the experimental protocol used to generate "Diet A" and "Diet B" in Figure 3B. Briefly Δ*moaA* mutant *E. coli* was cultured overnight at 37°C in 500μl of LB supplemented with 39 nanomoles of *Vc*MCP-bound Moco. The bacterial cells were then concentrated, washed, resuspended in LB, and seeded onto NGM to be fed to *moc-1(ok366) C. elegans* ("Diet B", green). The supernatant from this culture (spent LB+*Vc*MCP media) was then filtered (0.2 μm filter) to remove remaining bacterial cells and used to resuspend a separate culture of Δ*moaA* mutant *E. coli* that was grown only in LB. This was then seeded onto NGM to be fed to *moc-1(ok366) C. elegans* ("Diet A", purple). (B) *moc-1(ok366)* mutant *C. elegans* were synchronized at the L1 stage and cultured on Wild-type *E. coli*, Δ*moaA E. coli,* "Diet A", or "Diet B" (see Figure 3A for Diet A and B descriptions). For each experiment, animals were allowed to develop for 48 hours. Box plots display the median, upper, and lower quartiles while whiskers indicate minimum and maximum data points. Sample size (n) was 10 individuals assayed for each experiment.

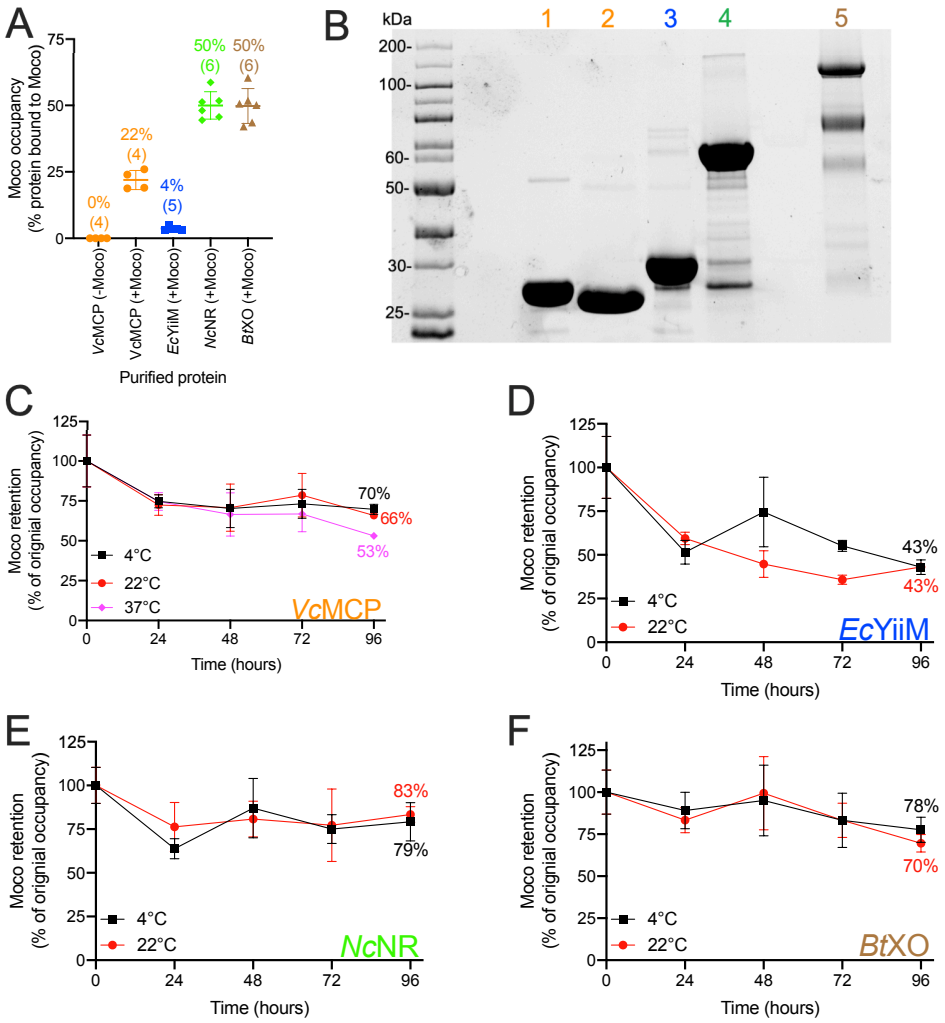
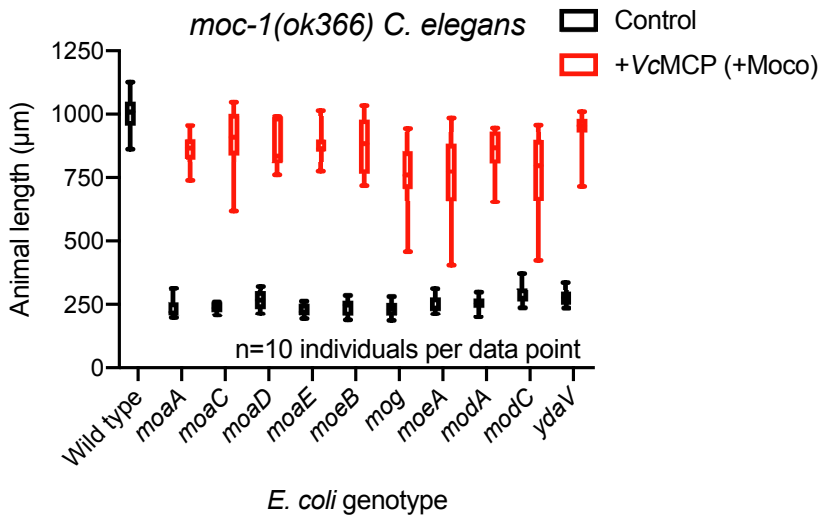


Figure 4: Stability of protein-bound Moco.

(A) Moco occupancies for purified *Ec*YiiM, *Vc*MCP, *Nc*NR, and *Bt*XO were determined by measurements of the Moco-derivative Form A and protein concentration. Moco occupancy is the percentage of protein molecules that are bound by a Moco prosthetic group. Moco occupancy was determined for *Vc*MCP purified from Moco-producing (+Moco) and Moco-deficient (-Moco) *E. coli.* The sample size (n) is displayed for each protein and each data point is individually presented with the mean and standard deviation. (B) Protein gel demonstrating the purity of [1] *Vc*MCP purified from Moco-deficient *E. coli*, [2] *Vc*MCP, [3] *Ec*YiiM, and [4] *Nc*NR purified from Moco-producing *E. coli*, and [5] *Bt*XO purified from bovine milk (Sigma-Aldrich). The gel displays all protein using the TGX Stain-Free system (Bio-Rad). (C-F) The amount of stable Moco retained by (C) *Vc*MCP, (D) *Ec*YiiM, (E) *Nc*NR, and (F) *Bt*XO was determined over 96 hours at 4°C (black) or 22°C (red). Moco retention of *Vc*MCP was also assessed at 37°C (pink). The Y-axis displays the Moco retention as a percentage of the original Moco occupancies (time 0) presented in Figure 4A. The sample size (n) is 3-6 replicates per protein and time point. The mean and standard deviation are displayed.

483 **Supplemental Materials:**



484

485 **Figure S1**: *E. coli* Moco-biosynthetic enzymes are not necessary for uptake of supplemental
486 protein-bound Moco by *C. elegans*.
487 *moc-1(ok366)* mutant *C. elegans* were synchronized at the L1 stage and cultured on wild-type
488 or mutant *E. coli* for 72 hours. Each growth assay performed with mutant *E. coli* was done with
489 or without 3.3 nanomoles of supplemental Moco bound to *Vc*MCP. The Y axis shows animal
490 length (µm), where 1,000µm roughly corresponds with fertile adulthood and 250µm roughly
491 corresponds to the L1 stage. Box plots display the median, upper, and lower quartiles while
492 whiskers indicate minimum and maximum data points. Sample size (n) was 10 individuals
493 assayed for each experiment.

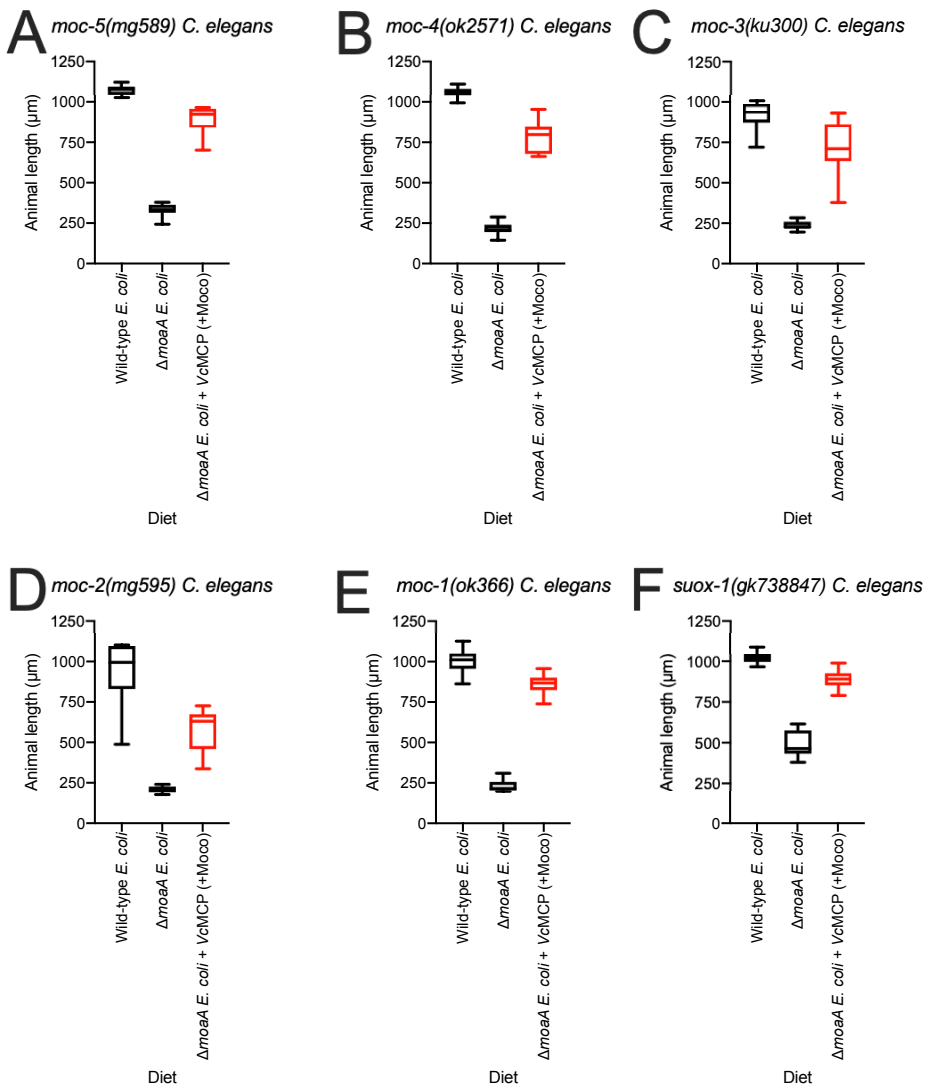


Figure S2: *C. elegans* Moco-biosynthetic enzymes are not necessary for uptake of supplemental protein-bound Moco.

(A) *moc-5(mg589),* (B) *moc-4(ok2571),* (C) *moc-3(ku300),* (D) *moc-2(mg595),* (E) *moc-1(ok366)* and (F) *suox-1(gk738847)* mutant animals were cultured from synchronized L1 larvae for 72 hours on wild-type, Δ*moaA E. coli,* or Δ*moaA E. coli* supplemented with 3.3 nanomoles of Moco bound to *Vc*MCP. The Y axis shows animal length (µm), where 1,000µm roughly corresponds with fertile adulthood and 250µm roughly corresponds to the L1 stage. Box plots display the median, upper, and lower quartiles while whiskers indicate minimum and maximum data points. Sample size (n) was 10 individuals assayed for each experiment.