Structural basis of kynurenic acid 3-monooxygenase inhibition

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Inhibition of kynurenine 3-monooxygenase (KMO), an enzyme in the eukaryotic tryptophan catabolic pathway (that is, kynurenine pathway), leads to amelioration of Huntington’s-disease-relevant phenotypes in yeast, fruitfly and mouse models1–5, as well as in a mouse model of Alzheimer’s disease6. KMO is a flavin adenine dinucleotide (FAD)-dependent monooxygenase and is located in the outer mitochondrial membrane where it converts L-kynurenine to 3-hydroxykynurenine. Perturbations in the levels of kynurenine pathway metabolites have been linked to the pathogenesis of a spectrum of brain disorders, as well as cancer7–9 and several peripheral inflammatory conditions9. Despite the importance of KMO as a target for neurodegenerative disease, the molecular basis of KMO inhibition by available lead compounds has remained unknown. Here we report the first crystal structure of *Saccharomyces cerevisiae* KMO, in the free form and in complex with the tight-binding inhibitor UPF 648. UPF 648 binds close to the FAD cofactor and perturbs the local active-site structure, preventing productive binding of the substrate L-kynurenine. Functional assays and targeted mutagenesis reveal that the active-site architecture and UPF 648 binding are essentially identical in human KMO, validating the yeast KMO–UPF 648 structure as a template for structure-based drug design. This will inform the search for new KMO inhibitors that are able to cross the blood–brain barrier in targeted therapies against neurodegenerative diseases such as Huntington’s, Alzheimer’s and Parkinson’s diseases.

There is great interest in the causative role of kynurenine pathway metabolites in neurodegenerative disorders such as Huntington’s and Alzheimer’s diseases6. Several of these metabolites are neuroactive: quinolinic acid (QUIN) is an excitotoxin10,11, 3-hydroxykynurenine (3-HK) generates free radicals12, xanthurenic and cinnabarinic acids activate metabotropic glutamate receptors13,14 and kynurenic acid (KYNA) is a neuroprotectant6. KMO lies at a critical branching point in the pathway between the synthesis of 3-HK/QUIN and KYNA (Fig. 1a) and its activity has a role in the neurotoxic and neuroprotective potential of the pathway. In the brain, KMO is expressed at low levels in neurons15 and is predominantly expressed in microglia16, the resident immune cells of the CNS, suggesting a link between KMO function and inflammatory processes in the brain.

Inhibition of KMO activity leads to amelioration of several disease-relevant phenotypes in yeast, fruitfly and mouse models1–5. Increased levels of KYNA relative to neurotoxic metabolites seem critical for this protection. Restoring endogenous levels of 3-HK to fruitflies lacking KMO activity eliminates this neuroprotection1, highlighting beneficial effects of 3-HK reduction due to KMO inhibition. In addition, pharmacological inhibition of KMO is neuroprotective in animal models of cerebral ischaemia17,18, reduces dystonia in a genetic model of paroxysmal dyskinesia19, improves levodopa-induced dyskinesia in parkinsonian monkeys20 and extends lifespan in a mouse model of cerebral malaria21. Therefore, inhibition of KMO activity is an attractive therapeutic strategy for several acute and chronic neurological diseases22.

Despite interest in targeting KMO only a few potent inhibitors are available, and none appreciably penetrate the blood–brain barrier in...
Table 1 | Kinetic and dissociation constants for human, *S. cerevisiae* and active-site variants of KMO

| Enzyme                     | \(K_{i app}\) UPF 648 (nM) | \(K_i\) UPF 648 (nM) |
|----------------------------|-----------------------------|-------------------|
| hKMO*                      | 56.7 ± 8                    | –                 |
| ScKMO(A394)                | 74 ± 14                     | 0.14 ± 0.01       |
| ScKMO(A394, R83A)           | –                           | 3.1 ± 0.2         |
| ScKMO(A394, R83M)           | –                           | 3.2 ± 0.2         |

* Dissociation constant \((K_i)\) for the human (h)KMO-UPF 648 complex could not be determined by fluorescence emission owing to low KMO expression yield.
† Kinetic assays to calculate the apparent inhibition constant \((K_{i app})\) of S. cerevisiae (Sc)KMO variants by UPF 648 could not be carried out because enzyme activity was substantially compromised after mutagenesis. As an alternative, fluorescence-emission measurements were performed to determine \(K_i\) by ligand perturbation of flavin fluorescence.

mutagenesis. As an alternative, fluorescence-emission measurements were performed to determine \(K_i\) of UPF 648 could not be carried out because enzyme activity was substantially compromised after mutagenesis. As an alternative, fluorescence-emission measurements were performed to determine \(K_i\) by ligand perturbation of flavin fluorescence.

adult animals\(^{1,2,2}\). One of these, UPF 648, has a half-maximum inhibitory concentration (IC\(_{50}\)) of 20 nM and provides protection against intrastriatal QUIN injections in kynurenine aminotransferase (KATII, also known as AADAT)-deficient mice\(^2\). UPF 648 treatment also shifts kynurenine pathway metabolism towards enhanced neuroprotective KYNA formation\(^{22,24}\), and ameliorates disease-relevant phenotypes in a fruitfly model of Huntington’s disease\(^4\). That known inhibitors do not cross the blood–brain barrier is an impediment to KMO-targeted drug discovery. KMO structures in complex with tight-binding inhibitors are required to design small-molecule inhibitors that can penetrate the blood–brain barrier. With this in mind, we determined the crystal structure of yeast KMO complexed with UPF 648. This enzyme–inhibitor structure can now be used to help develop new inhibitors of highly related human KMO.

We expressed full-length human KMO using the insect cell baculovirus system, which yielded small quantities (0.5 mg per litre of culture) of detergent-solubilized active KMO. The recombinant form had similar kinetic constants to native KMO from pig liver mitochondria\(^{25}\); UPF 648 binds tightly to recombinant KMO (inhibition constant \((K_i)\) = 56.7 nM). Poor stability and low expression yields, however, prevented crystallization. We therefore turned to *S. cerevisiae* KMO, which is related to human KMO (38% identity and 51% similarity). Expression of full-length *S. cerevisiae* KMO yielded a protein fragment (KMO(A396)) with a lower molecular weight than anticipated. Electrospray ionization mass spectrometry indicated proteolytic cleavage at residue 396. Subsequently, we isolated a KMO(A394) (residues 394–460 deleted) version of the enzyme engineered by site-directed mutagenesis (Methods) to define the cleavage point before crystallization (Supplementary Fig. 1 and Supplementary Table 1). The KMO(A394) enzyme was active ( Supplementary Figs 2 and 3), generated authentic 3-HK in high-performance liquid chromatography (HPLC)-based assays (Fig. 1b) and was inhibited by UPF 648 (\(K_i = 74\) nM) with a potency similar to that seen with human KMO (Fig. 1b and Table 1).

The structure of the proteolyzed form of yeast KMO (KMO(A396)) was determined using selenomethionine single anomalous diffraction (Protein Data Bank (PDB) codes 4J2W and 4J31). We also solved structures of KMO(A394) to 1.85 Å resolution. The final model contains residues 1–97 and 101–390 and the bound FAD cofactor. Both crystal forms contain a putative KMO dimer in the asymmetric unit (Fig. 2a). The KMO fold is similar to other flavin-dependent hydroxylase structures\(^{26,27}\), with highest structural similarity to 2-methyl-3-hydroxy pyridine-5-carboxylic acid oxy genase\(^2\) (root mean square deviation, 2.3 Å over 310 Cα atoms; overall sequence identity, 16%; Q-score, 0.43; Z-score, 15.0). An overlay of individual KMO monomers reveals variation in the position of the carboxy-terminal \(z\)-helix, with most monomers showing disorder beyond residue 380. The linker region following the second strand of the antiparallel \(\beta\)-sheet involved in substrate binding is disordered, with large variations in the positions of residues 96–97 and 101–104. The relative position of the FAD-binding domain and six-stranded antiparallel \(\beta\)-sheet domain is subject to minor variation, reminiscent of domain motion coupled to substrate binding in other members of this family\(^26\).

In absence of substrate, flexibility in relative positioning of both domains flanking the KMO active site is also reflected in distinct conformations observed for residues lining the active site (Fig. 3). The face of the FAD is connected to solvent by a narrow water-filled cavity that runs perpendicular to the active-site cleft (Supplementary Fig. 4). A structural water is located above the FAD C4a, mimicking the position of the C4a–peroxide intermediate formed upon reaction with oxygen. The dimethyl benzene moiety of the FAD isoxazoline is protected from solvent by Lys 48 and the conserved residue Tyr 195 (Supplementary Fig. 4). In absence of large protein rearrangements, this suggests that a ‘waving flavin’ motion as demonstrated in other FAD-dependent monoxygenases\(^2\) is unlikely to occur in KMO during turnover.

We were unable to obtain a KMO complex with L-kynurenine (L-KYN) but succeeded in co-crystallizing with UPF 648 (Supplementary Table 3, PDB code 4J36). The asymmetric unit contains a putative KMO dimer with one monomer containing UPF 648 bound in the active site, adjacent to the FAD re-face (Figs 2b and 3). The UPF 648 carboxylate is bound by conserved residues Arg 83 and Tyr 97 whereas the aromatic dichlorobenzene moiety is flanked by several hydrophobic residues.
(Leu 221, Met 230, Ile 232, Leu 234, Phe 246, Pro 321, Phe 322), which are conserved in many KMO enzymes. UPF 648 binding induced structural changes in the enzyme, notably reorientation of the Pro 321–Gln 325 loop flanking the re-side of the FAD. A minor reorientation in the position of the six-stranded antiparallel β-sheet domain with respect to the flavin-binding domain is also evident. These changes result in increased disorder of the C-terminal α-helix, which is only visible up to Arg 359 (Fig. 2b). Reorientation of the Pro 321–Gln 325 loop is a consequence of the active site adapting to the presence of vicinal chloride substituents in UPF 648, neither of which have a counterpart in l-KYN. To provide sufficient space for both chlorides, Phe 322 moves away from the active site, effectively occupying a position previously taken by Tyr 323. The Pro 321–Gln 325 loop reorients to compensate for the altered Phe 322 position. This loop lines the postulated oxygen-binding site above the re-side of the FAD, which is effectively destroyed on binding UPF 648. Binding UPF 648 was found to accelerate hydrogen peroxide formation by a factor of ~20-fold compared to reactions in absence of UPF 648 (Methods). This indicates a destabilization of the flavin C4a–hydroperoxide intermediate formed in the natural catalytic cycle of flavin monooxygenases in the presence of UPF 648 (Supplementary Table 2).

The chemical similarity of UPF 648 and l-KYN allowed modelling of l-KYN in the KMO active site (Methods). Modelling suggests that l-KYN is bound similarly, but without effect on the Pro 321–Gln 325 loop. The aromatic substrate moiety is located in the conserved hydrophobic pocket (residues Leu 221, Met 230, Ile 232, Leu 234, Phe 246, Pro 321, Phe 322) on the re-face of the flavin (Fig. 3). Additional polar contacts are formed between the conserved Glu 325 and the l-KYN carbonyl group, and between the substrate aniline nitrogen atom and the FAD O4 atom. Whereas the amino acid carboxylate is bound by Arg 83 and Tyr 97, the amino group is devoid of direct interactions with protein in the model. An additional salt bridge may be made between the l-KYN amine and the side chain of Glu 102, which is located in a highly flexible region of KMO. However, this residue is often replaced by a glutamine in other KMO enzymes (Supplementary Table 2).

**Figure 3** | The *Saccharomyces cerevisiae* KMO active site. Left panel shows an overlay of free enzyme structures obtained from various crystal forms, right panel depicts the KMO–UPF 648 interaction and middle panel shows a model of the KMO–l-KYN complex. Electron density is shown for UPF 648 ($2F_o - F_c$ contoured at 1σ).

**Figure 4** | Mechanism and importance of Arg 83. a, The proposed KMO l-KYN-hydroxylation mechanism based on the *S. cerevisiae* KMO–l-KYN model. After hydroxylation, the intermediate rearranges to form the 3-HK product. b, Enzymatic activity comparison between wild-type KMO and Arg 83 mutants. Enzyme activity is significantly reduced following mutation (~29% and <1% of wild-type activity for Ala 83 and Met 83 mutant KMO enzymes, respectively). Inset, measurement of binding constant for KMO inhibitor UPF 648 by ligand perturbation of flavin fluorescence emission. Titration of UPF 648 to 5 µM wild-type KMO (closed squares), R83M (closed triangles) and R83A (closed circles) mutants resulted in perturbation of 520 nm fluorescence emission. Fluorescence changes at 520 nm as function of UPF 648 concentration fitted to the Morrison equation yields an observed $K_d = 137.8 \pm 8$ nM for wild type, $K_d = 3.1 \pm 0.2$ µM for R83M and $K_d = 3.2 \pm 0.2$ µM for R83A enzymes. Error bars represent the standard deviation of three replica points. AU, arbitrary units.
Fig. 5), suggesting that this interaction is not critical for enzyme activity. The model places the substrate C3 atom adjacent to the flavin C4a, where it is poised to attack the flavin C4a-peroxide intermediate (Fig. 4a).

All residues implicated by the KMO–L-KYN model as being involved in L-KYN binding are conserved across KMOs (Supplementary Fig. 5). We validated this model by mutating residue Arg83 (replaced by Ala83 and Met83) and performing inhibitor binding/kinetic assays (Fig. 4b). Enzyme activity is compromised following mutation (25% and <3% of wild-type activity for Ala83 and Met83 mutant KMO enzymes, respectively) as predicted by the KMO–l-KYN model. Mutation led to ~20-fold increase in dissociation constant ($K_d$) for the KMO–UPF 648 inhibitor complex, which implicates Arg83 in inhibitor binding.

Elucidation of the KMO crystal structure in free form and in complex with an established inhibitor is a major breakthrough for new KMO inhibitor design. This will permit docking screens using virtual compound libraries that may ultimately identify novel inhibitor scaffolds. Such studies will enable the design of new inhibitors that possess the selectivity and affinity to open up new opportunities for therapeutically critical intervention. This, critically, should inform the development of brain-penetrant KMO inhibitors. The KMO inhibitor Ro 61–8048 and prodrug JM6 have shown preclinical promise in animal models of neurodegeneration2,4,16–20, but they do not appreciably penetrate the blood–brain barrier. These compounds probably confer neuroprotection3,30. As this neuroprotection seems downstream neurotoxic metabolite levels3, delivery of KMO inhibitors directly to the CNS should provide critical additional therapeutic efficacy and broaden the scope of disorders amenable to targeting.

METHODS SUMMARY

Discrimination assays. Human and S. cerevisiae KMO were produced using baculovirus and Escherichia coli expression systems, respectively. HPLC was used to monitor inhibition of KMO by UPF 648 by monitoring formation of product 3-HK across a range of inhibitor UPF 648 concentrations (0–2 μM). Flavin fluorescence-emission measurements were used to determine $K_d$ for KMO ligands through ligand perturbation of the fluorescence of enzyme-bound FAD. Detailed protocols can be found in Methods.

Crystallization and diffraction data collection. Crystals of S. cerevisiae KMO were obtained using the sitting-drop vapour-diffusion technique. Details describing the crystallography can be found in Methods. Diffraction data from S. cerevisiae KMO crystals were collected from single cryo-frozen samples at the Diamond Light Source, Harwell, UK. A single-wavelength anomalous dispersion data set at the selenium edge was used for initial phase determination. Data collection and processing details are presented in Supplementary Table 3.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions N.S.S., F.G., D.L. and T.F.O. initiated the project, designed experiments, analysed data and wrote the manuscript; M.A. cloned purified and crystallized proteins and performed biochemical assays; C.L. crystallized proteins, collected and processed diffraction data; D.J.H. developed and analysed some of the biochemical assays; P.L. performed molecular modelling of l-KYN binding.

Author Information Atomic coordinates and structure factors have been deposited in the Protein Data Bank (http://www.pdb.org) under accession numbers 4J2W, 4J31, 4J33, 4J36 and 4J34. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to N.S.S. (nigel.scrutton@manchester.ac.uk).
**METHODS**

Cloning, expression and purification of human full-length KMO. The gene encoding full-length human KMO was synthesized (GeneScript) and codon optimized for overexpression in mammalian cells. The gene was subcloned into the baculovirus transfer vector pAcGHLT-A—glutathione S-transferase as a Ndel–EcoRI fragment and transfected into Hi5 cells along with linearized baculovirus using the following sense and antisense primers: 5′-GGGATATGCATGGAGACAGAGGAA-3′; 5′-CCGAATTCCTACCGGCTAGC GTTTCGT-3′. Hi5 cells (1.5 × 10⁷) were infected with recombinant virus for 72 h at 28 °C. A WAVE Bioreactor System (GE Healthcare Life Sciences) was used to grow batches of 5-litres of cell culture. Cultured cells were lysed in 20 mM potassium phosphate buffer, pH 7.5, 10% glycerol, 0.5% n-dodecyl β-D-maltoside (DDM), 150 mM NaCl, 7 mM 2-mercaptoethanol and 50 μM FAD supplemented with protease inhibitors (Sigma–Aldrich). Soluble lysate was incubated with 3 ml pre-equilibrated glutathione uniflow resin (Clontech). The resin was then packed and washed with buffer B (20 mM potassium phosphate buffer, pH 7.5, 10% glycerol, 0.012% DDM, 150 mM NaCl, 7 mM 2-mercaptoethanol, 50 μM FAD). Fractions (0.5 ml) were eluted with buffer C (buffer B + 33 mM glutathione) and fractions containing KMO pooled, concentrated and loaded onto a Superdex 200 (10/30) size-exclusion chromatography column. Pure KMO was pooled and stored at −80 °C.

Cloning, expression and purification of *S. cerevisiae* KMO. A synthetic gene (BN44, UniProtKB accession number P38169) encoding *S. cerevisiae* KMO was codon optimized for overexpression in *E. coli* (GeneScript). The gene was subcloned into pET15b and pET24b (Merck) as a Ndel–XbaI fragment for overexpression in *E. coli* strain BL21(DE3). A deletion variant of KMO (*KMO*(A–394)) and single–amino-acid variants of this deletion (*KMO*(A394, R83A) and *KMO*(A394, R83M)) were generated by site-directed mutagenesis (QuickChange, Stratogene, Agilent Technologies). Sense and antisense primers are shown in Supplementary Table 1. Amplification conditions were as follows: denaturation at 95 °C for 30 s, followed by cycles of amplification at 95 °C (30 s), 55 °C (60 s) and 68 °C (7 min). PCR products were incubated for 1 h to digest methylated template DNA and then transformed into XL1-Blue supercompetent cells (Stratagene, Agilent Technologies). Transformed bacterial colonies were isolated and mutated genes were identified by DNA sequencing (Eurofins MWG Operon) to verify presence of desired, and lack of spurious changes, to the DNA sequence. Transformed bacterial cells were grown overnight at 37 °C in lysogeny broth (LB; 0.75 l) containing 100 μg ml⁻¹ ampicillin. Cultures were grown at 37 °C to an absorbance of 0.3, and the temperature was then reduced to 27 °C. Cells were induced by 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and grown (16–20 h) at 27 °C. Cells were lysed in buffer A (20 mM potassium phosphate buffer, pH 7.5, 10% glycerol, 300 mM NaCl, 50 μM FAD) containing protease inhibitors. Soluble lysate was loaded onto a 5 ml HisTrap FF Ni Sepharose 6 Fast Flow column (GE Healthcare) pre-equilibrated with buffer A. Protein was eluted using a 50–250 mM imidazole linear gradient. Yellow fractions containing partially purified KMO were pooled and diluted tenfold in buffer B to reduce the NaCl concentration. Protein was concentrated and chromatographed using a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with buffer C (25 mM ammonium acetate buffer, pH 7.0, 150 mM NaCl, 7 mM 2-mercaptoethanol). Pure fractions were collected and stored at −80 °C.

Incorporation of selenomethionine into *S. cerevisiae* KMO. Incorporation of selenomethionine-labelled protein was achieved by inhibiting methionine biosynthesis shortly before induction of KMO expression by adding high concentrations of isoleucine, leucine, phenylalanine, lysine and threonine to the cell culture. Transformed *E. coli* cells containing the *S. cerevisiae* KMO expression plasmid were grown in LB media at 37 °C to late exponential phase, collected and then suspended in M9 minimal media and grown until mid-log phase. At that point lysine, phenylalanine, threonine (100 mg l⁻¹ each) and selenomethionine, isoleucine, leucine and valine (50 mg l⁻¹ each) were added to the culture and induced 15 min after addition of amino acids with 0.1 mM IPTG. The culture was grown for 12–16 h. Purification of selenomethionine-incorporated *S. cerevisiae* KMO was as described for conventional *S. cerevisiae* KMO.

**Enzyme assays.** Steady-state kinetic parameters for KMO were obtained by initial rate measurements of enzyme activity. KMO catalyses the NADPH-dependent hydroxylation of L-KYN to 3-HK and the enzymatic reaction can be monitored by following the decrease in absorbance of NADPH at 340 nm. Rate assays were carried out with 1 cm path-length quartz microcuvette at 37 °C for human KMO and 30 °C for *S. cerevisiae* KMO. Pure enzyme (0.2–1.0 μM) was added to 200 μl reaction buffer (20 mM potassium phosphate buffer, pH 8.0, 7 mM 2-mercaptoethanol) containing different concentrations of NADPH and t-KYN (Sigma–Aldrich) and the time-dependent absorbance change at 340 nm was recorded using a using a UV–Vis Cary Eclipse spectrophotometer (Agilent Technologies). Assays at each reaction condition were performed in triplicate. Apparent Michaelis constants (Kₘ) for t-KYN and NADPH were determined by varying the concentration of the first substrate at a constant concentration of the second substrate and vice versa. Reaction data were fitted to the standard Michaelis–Menten equation using Origin Software (OriginLab).

KMO inhibition by UPF648 was measured by monitoring the formation of the product 3-HK by C₁₈ reversed-phase HPLC. Enzyme (100 nM) was incubated at 37 °C in 1 ml reaction buffer (20 mM potassium phosphate buffer, pH 8.5, 7 mM 2-mercaptoethanol) with 2 mM NADPH, 500 μM t-KYN and different concentrations (0–2 μM) of UPF68. The reaction was stopped with 8% trifluoroacetic acid added at different time points during the assay. Precipitated protein was removed by centrifugation, and the supernatant was analysed by C₁₈ reversed-phase HPLC equilibrated with 50 mM ammonium acetate, pH 3.0, containing 1% methanol and 0.1% heptanesulfonic acid; elution from the column was with 50 mM ammonium acetate, pH 4.5, containing 5% methanol and 0.5% heptanesulfonic acid, and monitored at 229 nm. 3-HK was quantified by comparison with a 3-HK calibration curve. Each experiment was performed in triplicate. Data were fitted to the Morrison equation:

\[
\frac{v_0}{v_i} = 1 - \frac{(E + I + K_i) - \sqrt{(E + I + K_i)^2 - 4EI[I]}}{2[E]}
\]

in which \(v_0\) is the enzyme activity without inhibitor, \(v_i\) is the enzyme activity in the presence of a ligand concentration \(I\), \(E\) is the enzyme concentration, \(I\) is the inhibitor concentration. 

Ligand-binding assays. Fluorescence-emission measurements were performed using a Cary Eclipse Fluorimeter (Agilent Technologies) to determine enzyme–ligand \(K_b\) for KMO ligands, exploiting ligand perturbation of flavin (enzyme–bound FAD) fluorescence. Excitation light was provided from a xenon light source and excitation and emission slit widths were 5 nm. The perturbation in FAD fluorescence emission was followed at 520 nm using an excitation wavelength of 450 nm and excitation spectra were recorded from 470 to 700 nm. t-KYN and UPF648 were titrated into a 5 μM solution of KMO (20 mM potassium phosphate buffer, pH 8, 50 mM NaCl, 7 mM 2-mercaptoethanol) at 25 °C. \(K_b\) was calculated by fitting data for the difference in fluorescence emission measured for KMO–ligand complexes and free KMO at 520 nm divided by the maximum emission versus the ligand concentration (equation (2)). This equation is a variation of Morrison equation used in equation (1) and is used for tight-binding ligands or when \(K_b\) values are similar in magnitude to the concentration of enzyme used, in which case substantial amounts of both enzyme and ligand are consumed in forming the EX complex as the titration progresses. All data fitting was done using Origin Software (OriginLab).

Hydrogen peroxide quantification. Measurements to observe the formation of hydrogen peroxide from the AKMO-394–UPF648 inhibitor complex were performed using a horseradish peroxidase (HRP) assay. Steady-state assays were performed as described above to determine the NADPH consumption rate in the presence and absence of an excess of UPF648 (30 μM). KMO (5 μM) was incubated with 150 μM NADPH, 150 μM t-KYN, 400 μM o-dianisidine and ~5 units HRP in the presence and absence of excess UPF648. The rate of oxidation of o-dianisidine by \(\text{H}_2\text{O}_2\) catalysed by HRP, was monitored at 440 nm (\(\Delta A_{440\text{nm}} = 11.600 \text{ M}^{-1} \text{cm}^{-1}\)). The rate of hydrogen peroxide production was compared to the rate of NADPH consumption.

**Crystallization of *S. cerevisiae* KMO.** Initial crystals of *S. cerevisiae* KMO (KMO(A396) and KMO(A396–Se)) were obtained by mixing 200 nl of 14 mg ml⁻¹ protein in 20 mM ammonium acetate, pH 7.0, 150 mM NaCl and 7 mM 2-mercaptoethanol (buffer A) with 200 nl of a reservoir solution containing 0.1 M sodium acetate, pH 5.5, and 35% isopropanol. A second, more readily reproducible, crystal form was obtained with AKMO-394 and AKMO–394–HS. In both cases crystals were grown by mixing 200 nl of protein (buffer A) with 200 nl of a reservoir solution containing 0.1 M imidazole, pH 7.8, and 11% w/v polyethylene glycol at 277 K. The UPF complex (AKMO-394–UPF648) was also obtained as described above; however, before setting the tray the protein was stored at −20 °C.
pre-incubated with 1 mM UPF 648 for ~30 min. All trays were incubated at 277 K, with crystals forming over a period of ~72 h.

**Diffraction data processing, structure determination and refinement.** X-ray diffraction data were collected at Diamond Light Source and subsequently integrated and scaled using the program XDS\(^{31}\). Initial phases were obtained from a single-wavelength anomalous dispersion data set (S1) collected at the selenium edge. Selenium sites were located using PHENIX AutoSol\(^{32}\) yielding an electron density map that could be auto traced using PHENIX AutoBuild\(^{33}\). The resulting model was completed through iterative rounds of rebuilding in Coot\(^{34}\) and refinement in PHENIX\(^{35}\). All subsequent structures were solved by molecular replacement in Phaser\(^{36}\) using this initial selenomethionine-derived structure as the template. Structure validation with MolProbity\(^{37}\) was integrated as part of the iterative rebuild and refinement procedure.

**KMO-\(t\)-KYN modelling.** NAMD software\(^{38}\) was used to perform all molecular dynamics simulations of the KMO-\(t\)-KYN complex. Topology and parameters files for substrate and FAD were obtained using Antechamber program\(^{39}\) using AM1-BCC charges\(^{40}\). The complex model was then immersed in a periodic water box (TIP3P) and neutralized by adding \(\text{Na}^+\) ions. Several cycles of minimizations (steepest descent, 10,000 steps) and molecular dynamics simulations (50 K, 20 ps) were performed to equilibrate the model (backbone protein atoms were kept fixed). Then molecular dynamics simulations were performed (310 K, 1 ns) at a time step of 2 ps, with the protein backbone restrained on the X-ray structure conformation. Individual snapshots were extracted and minimized to assess the KMO-\(t\)-KYN interactions.

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