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Investigation into the mechanism of λ_{max} shifts and their dependence on pH for the 2-hydrazinopyridine derivatives of two copper amine oxidases

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Abstract

Copper amine oxidases (CuAO), from *Escherichia coli* (ECAO) and pea seedling (PSAO) were reacted with an excess of the hydrazine derivative 2-hydrazinopyridine (2HP) to form an initial, strongly absorbing adduct, (adduct 1; λ_{max} 420–430 nm) formed by the covalent binding of 2HP with the active site cofactor 2,4,5-trihydroxyphenylalanine quinone (TPQ). Thermal incubation of buffered solutions of adduct 1 (pH 5.65–10.7) or addition of KOH solution (giving a final pH of 13–15) led isosbestically to a dramatic λ_{max} shift yielding adduct 2 (λ_{max} 520–530 nm). For both ECAO and PSAO, an increase in pH resulted in increased formation of adduct 2 with concomitant loss of adduct 1. Maximum adduct 2 formation occurred at pH 9.84 in ECAO and at pH 10.7 in PSAO. Beyond these pH levels, adduct 2 formation occurred to a much lesser extent which was independent of pH, suggesting enzyme denaturation. It is proposed that the conversion of adduct 1 to adduct 2 occurs as a result of hydrazone to azo conversion mediated by loss of a single proton, possibly to the active site base. It is further postulated that adduct formation and subsequent deprotonation can be likened to the substrate and product Schiff base complexes in the reductive half cycle of copper/TPQ containing amine oxidases. As part of this study an extinction coefficient at 280 nm was determined for ECAO by gravimetric analysis. This yielded a value of 2.1×10^5 M^{-1} cm^{-1} giving rise to the need of a correction factor when estimating the protein concentration from an absorbance reading at 280 nm. Using the estimated molecular mass of 160 kDa for the homodimeric ECAO, a correction factor of 0.76 must be applied.

Keywords: Amine oxidase; Inhibition; pH Effects; Hydrazone-azo conversion

1. Introduction

Copper amine oxidases (CuAO) are a diverse family present in organisms as distinct as bacteria, fungi, plants and mammals [1–4]. These enzymes are responsible for the oxidative deamination of a wide range of amine substrates, from mono to poly amines and they display a broad substrate specificity as well as an intriguing stereoselectivity [5]. In 1990 the cofactor in CuAOs was identified as 2,4,5-trihydroxyphen-

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ylalanine quinone (TPQ) [6]. Indeed, the cofactor in *Escherichia coli* (ECAO) and the characterization of the enzyme have been the subject of a number of excellent studies [7–9]. TPQ is derived from a post-translationally modified tyrosine residue [10–12]. Cofactor identification involved labeling the bovine serum CuAO with [U–^{14}C]phenylhydrazine, which forms a covalent adduct with TPQ at the O5 position, proteolytic digestion, to isolate the derivatised cofactor, then NMR and mass spectroscopic analysis to identify the isolated adduct. Subsequently it has been shown that the *p*-nitrophenylhydrazine adducts of TPQ have distinctive visible absorbance spectra which have facilitated the definitive assignment of TPQ as the active site cofactor in other such quino-enzymes [11]. Interestingly, the absorbance maxima of the derivatised enzyme of 457–472 nm at pH 7.2 can be shifted to 577–587 nm in 1–2 M KOH [11,13]. Numerous studies have been undertaken to investigate the effects of different hydrazine-derivatives on the catalytic activity and spectrophotometric properties of CuAO in further attempts to elucidate the nature of substrate catalysis [14–17]. In particular, the recent crystal structure of 2-hydrazinopyridine (2HP) bound to ECAO has placed the chemistry in a structural context [18]. The 2HP is covalently linked to the 5 position of the TPQ ring (Fig. 1). The secondary amine group (N2) and the pyridine ring nitrogen (N3) of the adducted 2HP (inset, Fig. 1) are hydrogen bonded to Asp383, identified as the catalytic base in the reaction. The complex (studied at pH 7.2) is analogous to the substrate Schiff base formed during the reaction with substrate. The quinone/inhibitor moiety also interacts with other conserved elements of the active site, including Tyr369, Asn465 and the apical water ligand of the copper ion.

To investigate the nature of the absorbance maxima shift, and its significance to the mechanism of CuAO, a thorough investigation of the pH dependence of this shift has been undertaken using CuAOs from ECAO and pea seedling (*Pisum sativum*, PSAO) adducted with the hydrazine derivative 2HP. These results are considered in the context of the crystal structure of the 2HP/ECAO complex [18].

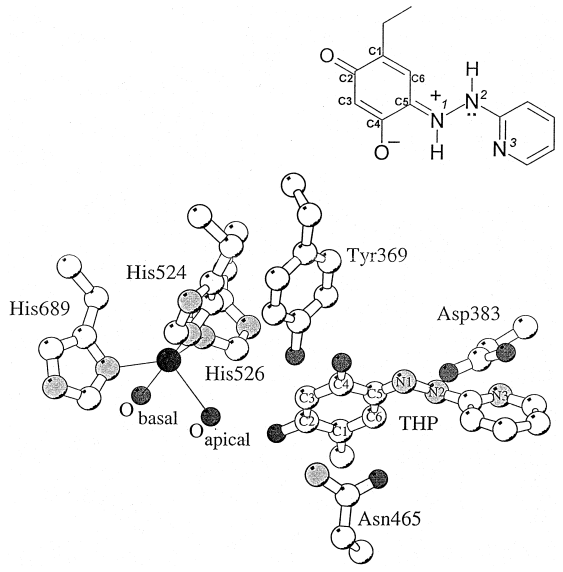

Fig. 1. The relationship between the quinone/2HP adduct (labeled THP), conserved residues and the copper site. The conserved residues directly interacting with the quinone/inhibitor are Tyr369, Asn465 and the catalytic base Asp383. The distorted square pyramidal arrangement of the copper ion and its ligands is displayed. White spheres correspond to carbon atoms (numbered in the TPQ cofactor, main picture and inset), light gray to nitrogen atoms, dark gray to oxygen atoms and black to the copper atom. O_{basal} and O_{apical} correspond to the two copper bound water molecules. Figure generated using MOLSCRIPT [19]. The inset shows a schematic representation of the TPQ–2HP adduct with the ring nitrogen (N3), neighboring secondary amine nitrogen (N2) and TPQ bound N (N1) of the 2HP moiety (as referred to in the text).

2. Materials and methods

2.1. Enzyme preparation

PSAO and ECAO purification and activity assay were performed as previously reported [20,21]. The purity of all enzymes used was assessed by SDS-PAGE analysis. Previously, ECAO concentrations had been determined by the method of Bradford [22]; for the purpose of this study ECAO concentration was determined by a gravimetric method. Here, 0.5 ml samples

of known specific activity and absorbance at 280 nm were extensively dialyzed vs. deionized water, frozen in liquid nitrogen and lyophphilized overnight in 1.5-ml Eppendorf tubes at −40°C. An accurately weighed amount of the freeze-dried protein, typically 3–5 mg, was then reconstituted in 5 ml of water, the volume remeasured and its specific activity and absorbance at 280 nm checked [23]. The ECAO used in these experiments was found to be >98% pure from analysis of the SDS-PAGE gels by a Flowgen AlphaImager™ 2000 gel scanner. PSAO concentrations were calculated using an extinction coefficient at 280 nm of 3×10^5 M^{-1} cm^{-1} [20].

Proteins were used, typically, in the range $(1.0–6.0) \times 10^{-6}$ M. All absorbance measurements were performed on a Shimadzu UV-2401PC UV–VIS recording spectrophotometer, thermostatted at 25.0°C.

2.2. Buffers

Enzyme was dialyzed overnight into the appropriate pH buffer; 2-*N*-morpholino ethanesulphonic acid, Mes (Sigma, reagent grade) was used between pH 5.5 and 6.0, sodium phosphate (BDH, Analar) was used between pH 5.70 and 8.00, 2-[*N*-cyclohexylamino]-ethanesulphonic acid, Ches, (Sigma, reagent grade) was used between pH 8.60 and 10.0, and sodium carbonate (BDH, Analar) was used between pH 10.3 and 10.7. Mes and Ches solutions were adjusted to the required pH by addition of 1 M NaOH (BDH) whereas the pH of sodium phosphate and sodium carbonate buffers were adjusted by mixing solutions of the mono- and disodium salts according to the method of Gomori [24]. Buffers were made up to 20 mM concentration in deionized water and adjusted to an ionic strength of 0.100 M using NaCl (Sigma). Initial and final pH of solutions were measured on a Radiometer (PHM62) pH meter fitted with a Russell CWR/32 combination Ag/AgCl reference/glass electrode.

2.3. 2HP solutions and adduct formation

Solutions of 2HP (dihydrochloride salt, Aldrich; used as supplied) were prepared in deionized water and suitably diluted on addition to the buffered enzyme solution to give a final concentration of between 1.5–2 equivalents which have been shown by previous titration experiments to saturate ECAO [23] yielding adduct 1; the corresponding excess of 2HP used with PSAO was between 2 and 3 equivalents [23]. Typically 2 μl aliquots of 2HP stock solution were added to 1 ml of enzyme solution. Formation of adduct 2, between pH 5.65 and 10.7 was performed by incubation of adduct 1 at 60°C for 30 min or until no further absorbance change was noted. For adduct 2 formation at pH > 10.7, adduct 1 was first formed in enzyme buffered at neutral pH and then pH jumped by the addition of a small aliquot of 10 M KOH (BDH, Analar) to give a final KOH concentration of ≥ 1 M; no thermal incubation was necessary for maximum conversion. Any slight precipitation was removed from solution by centrifugation prior to taking the spectrum.

2.4. Enzyme assays

Enzyme activity was measured spectrophotometrically using a coupled assay method [25]. For ECAO the substrate used was β-phenylethylamine [26] and for PSAO the substrate used was benzylamine [27].

3. Results

The dry weight determination of the extinction coefficient of ECAO is based on protein concentration, in mg/ml, as described above. The results give an extinction coefficient at 280 nm of 2.10×10^5 M^{-1} cm^{-1} making the application of a correction factor to absorbance readings at 280 nm necessary when estimating pro-

tein concentrations. Using a molecular mass for ECAO of 160 kDa, the correction factor is 0.76. A calculation of the extinction coefficient from the amino acid sequence, using the method of Gill and von Hippel [28] gave 2.43×10^5 M^{-1} cm^{-1} [29]. The latter value was not used in calculating protein concentrations as this does not take the protein microenvironment into account and the effects this may have on individual residues. Specific activity of ECAO was found to be relatively unaffected by the lyophilization procedure. A loss of no more than 4% occurred in the sample used, from 24 to 23 μmol mg^{-1} min^{-1} by the coupled assay method, described above.

UV–VIS spectra were recorded following the reaction of both ECAO and PSAO with 2HP. Two adducted species were identified with significantly differing λ_{max} values. Adduct 1 (λ_{max} 420–430nm) formed rapidly but converted to adduct 2 (520–530 nm) very slowly at 25°C, even at an elevated pH of 9.14. The time course of this reaction was measured over a 150 h period (Fig. 2). While the formation of adduct 2 was not complete there is a clear isosbestic point at 460 nm indicating that the two species are in equilibrium. The inset to Fig. 2 reveals a monophasic process further indicative of a single event, such as a deprotonation shown in Eq. (1).

$$[\text{TPQ–2HP}]\text{H}^+ \overset{K_a}{\rightleftharpoons} [\text{TPQ–2HP}] + \text{H}^+ \qquad (1)$$

where the acid dissociation constant is given by Eq. (2).

$$K_a = \frac{[\text{H}^+][(\text{TPQ–2HP})]}{[(\text{TPQ–2HP})\text{H}^+]} \qquad (2)$$

A similar study with PSAO also yielded similar results (data not shown).

More extreme conditions were employed to examine the extent to which the equilibrium between adducts 1 and 2 could be altered in favour of adduct 2 formation. Enzyme was incubated at various pH values at 60°C before a UV–VIS spectrum was recorded. The extent of

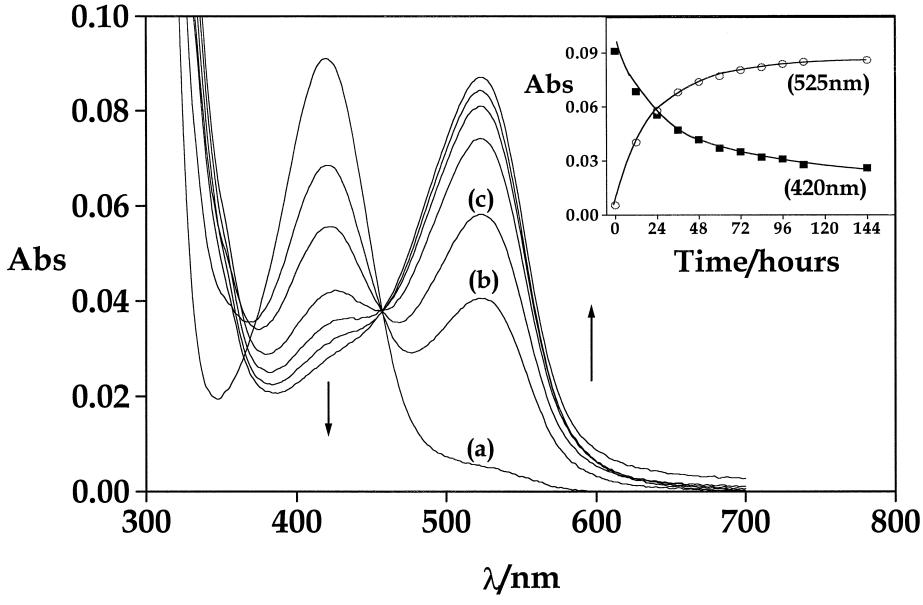

Fig. 2. Time dependent optical spectra showing the conversion of ECAO adduct 1 to adduct 2 (enzyme at 2.0×10^{-6} M) in 20 mM Ches buffer, pH 9.14, 25°C using a 50-fold excess of 2HP over enzyme. Spectra (a) at time = 0 h, (b) at time = 12 h, (c) at 24 h then at 24-h intervals afterwards. The reaction was terminated after ~150 h. The inset shows the λ_{max} increase of adduct 2 (525 nm, ○) and the decrease of adduct 1 λ_{max} (420 nm, ■).

Table 1
The variation in adduct 2 Abs_{max} to adduct 1 Abs_{max} ratio with pH (20 mM buffers) for the 2HP adduct of ECAO, after thermal incubation (at 60°C for 30 min or until no further absorbance change occurred) or KOH treatment of adduct 1. 2HP in 1.5–2 fold excess of ECAO. Protein concentration in the range (1.0–6.0) $\times 10^{-6}$ M, $I = 0.100$ M (NaCl)

Method of adduct 2 formation	Final pH	Ratio: adduct 2/ adduct 1
60°C heating	5.65	1.13
60°C heating	6.90	1.65
60°C heating	8.02	2.81
60°C heating	8.60	4.65
60°C heating	9.14	6.60
60°C heating	9.84	8.60
60°C heating	10.2	2.92
60°C heating	10.7	2.80
1 M KOH	13.0	3.01
1.66 M KOH	14.0	3.09
2.85 M KOH	15.0	3.10
2.85 M KOH	15.1	2.92

formation of adduct 2 was calculated as the ratio of absorbance values at the λ_{max} for both adducts (Tables 1 and 2). A significant effect due to pH was observed (Figs. 3 and 4). The ratio (see Table 1) increased to 8.6 at pH 9.84 for ECAO and 5.74 at 10.7 for PSAO, but no plateau was reached indicating that maximal conversion to adduct 2 was not achieved. Above

Table 2
The variation in adduct 2 Abs_{max} to adduct 1 Abs_{max} ratio with pH (20 mM buffers) for the 2HP adduct of PSAO, after thermal incubation (at 60°C for 30 min or until no further absorbance change occured) or KOH treatment of adduct 1. 2HP in 2- to 3-fold excess of PSAO. Protein concentration in the range (1.0–3.0) $\times 10^{-6}$ M, $I = 0.100$ M (NaCl)

Method of adduct 2 formation	Final pH	Ratio: adduct 2/ adduct 1
60°C heating	5.55	1.00
60°C heating	6.02	1.55
60°C heating	6.90	1.58
60°C heating	8.07	1.82
60°C heating	9.23	2.95
60°C heating	10.2	5.74
60°C heating	10.5	4.9
60°C heating	10.7	5.77
1.66 M KOH	14.0	2.86
2.85 M KOH	15.1	2.52

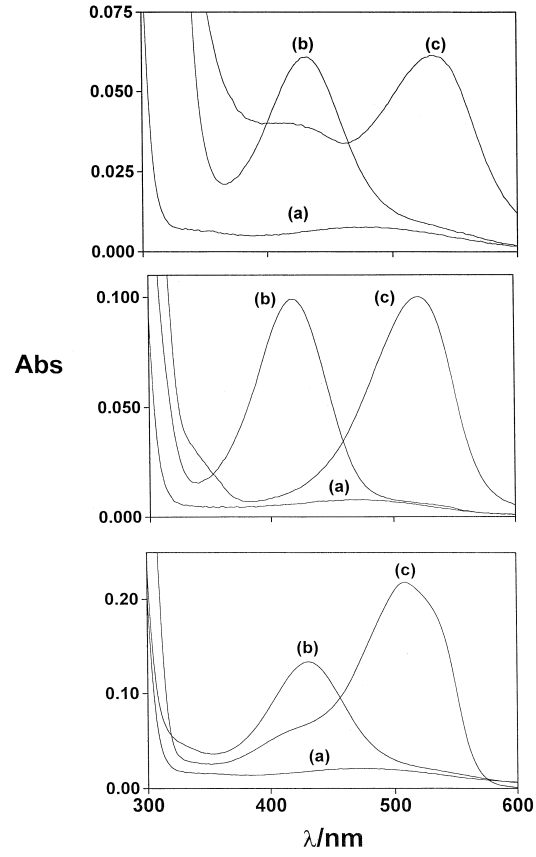

Fig. 3. Optical spectra of *E. coli* CuAO and 2HP derivatives. Where spectrum (a) corresponds to unadducted ECAO, (b) corresponds to adduct 1 and (c) to adduct (2). Spectra taken at pH 6.90, ECAO at 2.2×10^{-6} M (top) and pH 9.84 (middle), ECAO at 2.2×10^{-6} M, where adduct 2 was formed by thermal incubation at 60°C. The bottom set of spectra corresponds to neutral pH ECAO, 4.3×10^{-6} M, (a), adduct 1 at neutral pH (b) and adduct 2 (c) formed by addition of KOH solution to a final concentration of 2.85 M and pH of 15.1.

these pH values a constant ratio of 2.5 to 3.1 was observed for both ECAO (pH 10.2–15.1) and PSAO (> 10.7). It was not possible to determine an accurate pK_a value for Eq. (2) due to the absence of a high pH plateau region for both ECAO and PSAO systems. A second process may also account for the absence of a plateau.

It seemed likely that the sharp transition from increasing ratio to a lower constant ratio at high pH is due to protein denaturation. To test this, non-adducted ECAO samples were incubated in

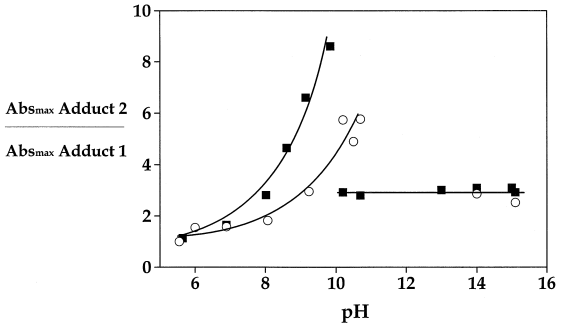

Fig. 4. Plot to illustrate the dependence of adduct 2 formation on pH. The proportion of adduct 2 formed and adduct 1 remaining is indicated by the ratio (Adduct 2 Abs_{max} / Adduct 1 Abs_{max}). These absorbance readings were taken from the adduct 2 spectrum of ECAO (■) or PSAO (○) after either heating buffered solutions of adduct 1 (pH 5.5–10.7) or treatment with KOH solution (pH 13–15.1).

various pH buffers for approximately 15 min before adjusting the pH to neutral for enzyme assay. The coupled assay system was used and the rate of oxidation of β-phenylethylamine by untreated enzyme at 30°C was 24 μmol mg^{-1} min^{-1}. For samples incubated at pH values up to 9.84 full enzyme activity was recovered. By contrast above pH 9.84 there was a decline in recovered activity with increasing pH. Similar experiments with PSAO using benzylamine as a substrate showed full activity recoverable following incubation at pH 10.7. For both enzymes incubation at extreme pH values of 13–15 prevented recovery of any activity. These studies suggest, as expected, that protein conformation is affected by high pH.

4. Discussion

Insight into the structures of the TPQ–2HP adducts 1 and 2 was obtained from the detailed analysis of the X-ray crystal structure of 2HP

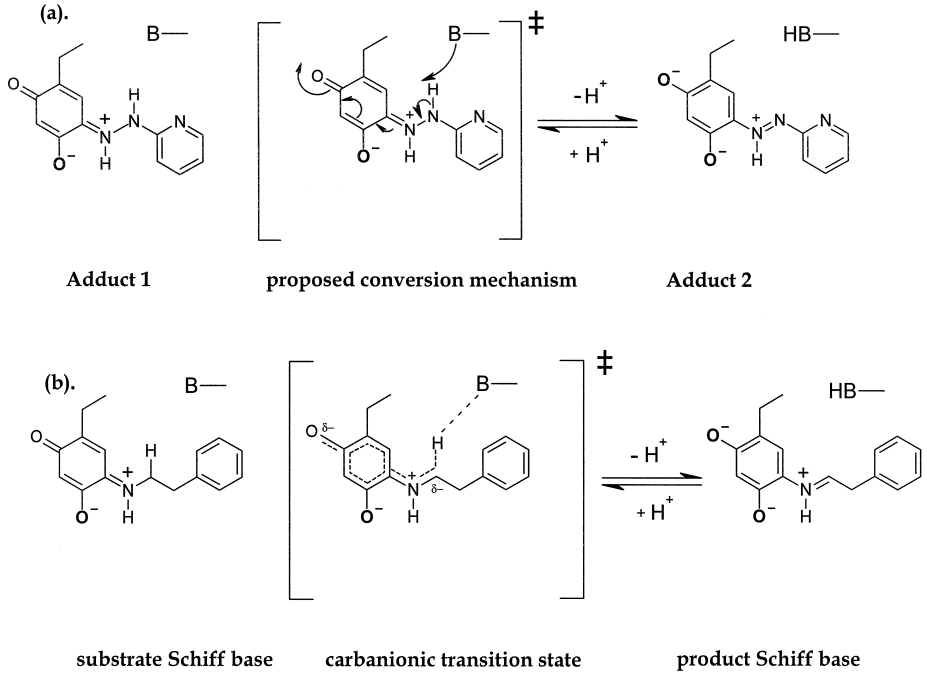

Fig. 5. (a) Proposed conversion mechanism of adduct 1 to adduct 2 in ECAO and PSAO, illustrating the single proton removal from N2 of the 2HP moiety by a base (B), possibly being of protein origin (Asp383, ECAO or Asp300 PSAO, the catalytic base) or by solvent. The corresponding mechanism (b) of substrate (β-phenylethylamine) to product Schiff base conversion, via a carbanionic transition state [30–32].

derivatised ECAO [18]. The crystal structure is likely to be that of adduct 1 as the crystals were grown in 0.1 M Hepes buffer, pH 7.2. The solution studies demonstrated that thermal incubation at around neutral pH is required to produce adduct 2 in 1.65-fold excess over adduct 1 (Fig. 3, top). The X-ray diffraction data obtained from 2HP-derivatised ECAO is therefore thought to be a hydrazone derivative.

It is further proposed that proton abstraction, most plausibly from the secondary amine group of 2HP results in a hydrazone to azo conversion (adduct 1 to adduct 2, respectively, Fig. 5). Hydrazone to azo tautomerism has been well characterised in synthetic systems, and there are many comparable features between the automeric reaction in synthetic systems and the conversion thought to occur at the active site of CuAOs. For example, it has been shown that λ_{max} shifts, of an isosbestic nature, occur as a result of the conversion of a hydrazone complex to an azo complex [33–35]. The conversion of adduct 1 to adduct 2, through the possible proton abstraction from the secondary amine group (N2) of 2HP, would result in a complex that is likely to be analogous to the product Schiff base intermediate that occurs as part of the natural reaction cycle when CuAOs react with their natural substrates (Fig. 5). The pK_a for the proposed conversion is high. An earlier study using *p*-nitrophenylhydrazine and a TPQ model found it to be 12.2 [11]. However, the possibility of a second group, involved in an acid/base capacity, cannot be excluded. This could account for the absence of a upper plateau at high pH (Fig. 4).

The data suggest that adduct 1, the putative hydrazone form of the ECAO–2HP complex, is the kinetic product and adduct 2 is the thermodynamic product, with a relatively high-energy transition state for their interconversion. An explanation for this phenomenon was sought by a detailed analysis of the 2HP-derivatised structure (Fig. 1). Examination of the electron density maps obtained from the underivatised crystal structures of ECAO reveal that the TPQ ring is free to rotate around the β carbon atom [21]. However the electron density maps obtained from crystals of ECAO derivatised with 2HP reveal well defined electron density for the complex. Therefore the TPQ–2HP complex is in a single dominant conformation within the crystal [18]. A π–π stacking interaction between Tyr381 and the pyridine ring of 2HP is thought to be important in the stabilisation of adduct 1. Previous studies using bovine serum amine oxidase (BSAO) have indicated that a single residue is responsible for proton abstraction from the substrate [36]. The crystal structure of the ECAO–2HP complex and mutagenesis experiments identified this residue as Asp383 in ECAO [18]. The corresponding base in PSAO is D300 [37] which is structurally conserved between ECAO and PSAO. The ECAO–2HP crystal structure demonstrated that this residue is capable of making two hydrogen bonds to the 2HP moiety of the TPQ–2HP complex. A normal hydrogen bond of 2.8 Å occurs between the δ2 oxygen of Asp383 and the N2 of 2HP. Intriguingly, there is a distance of only 2.5 Å separating the δ1 oxygen of Asp383 and the ring nitrogen of 2HP (N3) despite the presence of normal distance restraints between atoms during refinement. The latter distance suggests a strong bonding interaction between the δ1 oxygen of Asp383 and N3 of 2HP and demonstrates the presence of a proton shared between these atoms. This may play an important role in impeding the rate of conversion of adduct 1 to adduct 2. Based on previous findings [36] it is proposed that Asp383 is fully deprotonated when 2HP binds and both N1 and N2 of 2HP are protonated. Preliminary UV–VIS and proton NMR studies on 2HP alone indicate that a pK_a of 7.1 may be due to the N3 group [23].

These data suggest a possible model for the hydrazone to azo conversion implicated by this study for the 2HP complex with ECAO and PSAO. The ability of D383 to act as a base in ECAO (and abstract a proton from N2) would be reduced by the presence of the shared proton between N3 of the pyridine ring and the oxygen

of the acid group of D383. This is observed in the crystal structure of the complex at pH 7.2. The expected lower pK_a of the N3 position compared to the N2 position, suggests that this proton may initially be lost upon increasing the basicity of D383 and promote the abstraction of the proton from N2, leading to the formation of adduct 2. The crystal structure of 2HP/ECAO thought to represent adduct 1, shows that the two rings of TPQ and 2HP are not conjugated. The abstraction of a proton from N2 would result in a fully conjugated system (Fig. 5a), which is consistent with this being the thermodynamically favoured form. The large λ_{max} shift in the UV–VIS spectrum, from adduct 1 to adduct 2, supports this hypothesis. The removal of the N3 proton alone, which would not alter the overall conjugation, would not be expected to lead to such a large λ_{max} shift. The constant ratio of 2.5 to 3.1, between adduct 1 and adduct 2 at higher pH (Fig. 4), is probably due to denaturation of the protein. This would destroy the carefully controlled chemistry created by the protein active site microenvironment, leaving the 2HP/TPQ complex exposed to the solvent. In this case the solvent itself would act as the base.

Although this study is preliminary it gives a brief summary of our current understanding of this reaction and provides scope for further kinetic and thermodynamic studies involving different hydrazine inhibitors, experimental conditions and enzyme variants. The greater stability of 2HP (compared to those of phenylhydrazine and nitrophenylhydrazine) makes it a useful probe in these studies.

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