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Albino mutants of *Streptomyces glaucescens* tyrosinase

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Site-directed mutagenesis was used to determine the functional role of several residues of *Streptomyces glaucescens* tyrosinase. Replacement of His-37, -53, -193 or -215 by glutamine yields albino phenotypes, as determined by expression on melanin-indicator plates. The purified mutant proteins display no detectable oxy-enzyme and increased Cu lability at the binuclear active site. The carbonyl derivatives of H189Q and H193Q luminesce, with $\lambda_{max.}$ displaced more than 25 nm to a longer wavelength compared with native tyrosinase. The remaining histidine mutants display no detectable luminescence. The results are consistent with these histidine residues (together with His-62 and His-189 reported earlier) acting as Cu ligands in the *Streptomyces glaucescens* enzyme. Conservative substitution of the invariant Asn-190 by glutamine also gives an albino phenotype, no detectable oxy-enzyme and labilization of active-site Cu. The luminescence spectrum of carbonyl-N190Q, however, closely resembles that of the native enzyme under conditions promoting double Cu occupancy of the catalytic site. A critical role for Asn-190 in active-site hydrogen-bonding interactions is proposed.

INTRODUCTION

Tyrosinase (EC 1.14.18.1) is a copper mono-oxygenase that catalyses the *o*-hydroxylation of monophenols and the oxidation of *o*-diphenols to *o*-quinones (Mason, 1965). The widely occurring enzyme is responsible for formation of melanins and other pigments (Lerch, 1981). In recent years, the structure and reactivity of tyrosinases from *Neurospora crassa* and *Agaricus bisporus* have been extensively studied (Lerch, 1983; Schoot Uiterkamp & Mason, 1973). Spectroscopic data have led to a proposed reaction mechanism incorporating an electronically coupled binuclear Cu complex at the enzyme's active site (Winkler *et al.*, 1981; Lerch, 1988).

Tyrosinase shares several spectroscopic properties with the O_2-transport protein haemocyanin, notably the $O_2^{2-} \rightarrow Cu(II)_2$ charge-transfer band of the oxy-proteins (Wilcox *et al.*, 1985) and the luminescence of the carbonyl derivatives (Kuiper *et al.*, 1980*a*,*b*). The two proteins also share a highly conserved region of primary structure, the Cu_B region (Lerch *et al.*, 1986; Müller *et al.*, 1988), containing three invariant histidine residues. In the crystal structure of arthropodan haemocyanin from *Panilirus interruptus*, these histidines are seen to act as ligands to Cu_B (Gaykema *et al.*, 1984).

Conservation of the Cu_A-binding site is much poorer (Lerch *et al.*, 1986). The protein sequence containing the three Cu_A ligands of *P. interruptus* haemocyanin has only limited similarity to that of *N. crassa* tyrosinase (Lerch, 1978) and no detectable similarity to sequences of the enzyme from mammals (Kwon *et al.*, 1987; Müller *et al.*, 1988) or from the Gram-positive bacterium *Streptomyces glaucescens* (Huber *et al.*, 1985). However, Cu_A-binding-site sequence conservation has been noted between *S. glaucescens* and mammalian tyrosinases and haemocyanin from the mollusc *Helix pomatia* (Drexel *et al.*, 1986). This highly conserved region contains three histidine residues, which may constitute a Cu_A-binding site distinct from those found in *N. crassa* tyrosinase and arthropodan haemocyanins.

In our laboratory we use site-directed mutagenesis of the *S. glaucescens* tyrosinase gene in order to define the copper ligands and the role of other highly conserved residues. We have shown that His-62 and -189 are Cu ligands in this enzyme (Huber & Lerch, 1988). In the present work, four additional histidine residues were targeted as potential Cu ligands of *S. glaucescens* tyrosinase. The choice of histidine residues was based on alignment of the amino acid sequences mentioned above, augmented by evidence obtained from reaction-inactivation experiments with *N. crassa* tyrosinase (Dietler & Lerch, 1982). The effect of single His→Gln exchanges at these residues was investigated.

In addition, the importance of invariant residue Asn-190 was studied through its conservative substitution by glutamine. Asn-190 lies adjacent to Cu_B ligand His-189, and the analogous asparagine in the crystal structure of *P. interruptus* haemocyanin is seen to bridge the active-site Cu atoms (Gaykema *et al.*, 1984). Reactivity and physicochemical properties of the native and mutant enzymes are compared, with particular emphasis on the luminescence of the carbonyl derivatives.

MATERIALS AND METHODS

Bacterial strains and plasmids

The M13mp19 vector was propagated in *Escherichia coli* strains CSH50 and JM109 as previously described (Huber & Lerch, 1988). Subcloning vector pKL7 and shuttle vector pAH1, derived from pUC19 (Yanisch-Perron *et al.*, 1985) and the *Streptomyces* plasmids pIJ364 and pMEA4 (Kieser *et al.*, 1982; Hintermann *et al.*, 1985), were propagated in JM83 [*ara*, Δ*lacpro*, *strA*, *thi*, (Φ80, d*lacZ* M15)] in LB medium supplemented with ampicillin (70 μg/ml). Tyrosinase was expressed in *Streptomyces lividans* strain TK23, which is devoid of the structural gene for tyrosinase (Hintermann *et al.*, 1985).

Manipulations of *E. coli* and *Streptomyces* followed standard methods (Sambrook *et al.*, 1989; Hopwood *et al.*, 1985). To construct pKL7, the 2.2 kb *Kpn*I fragment of pMEA4 containing the complete *S. glaucescens* tyrosinase gene was ligated into *Kpn*I-opened pUC19 in which the *Sma*I–*Hin*dIII fragment of the polylinker had previously been removed and the sticky ends filled

Abbreviations used: L-dopa, 3,4-dihydroxy-L-phenylalanine.

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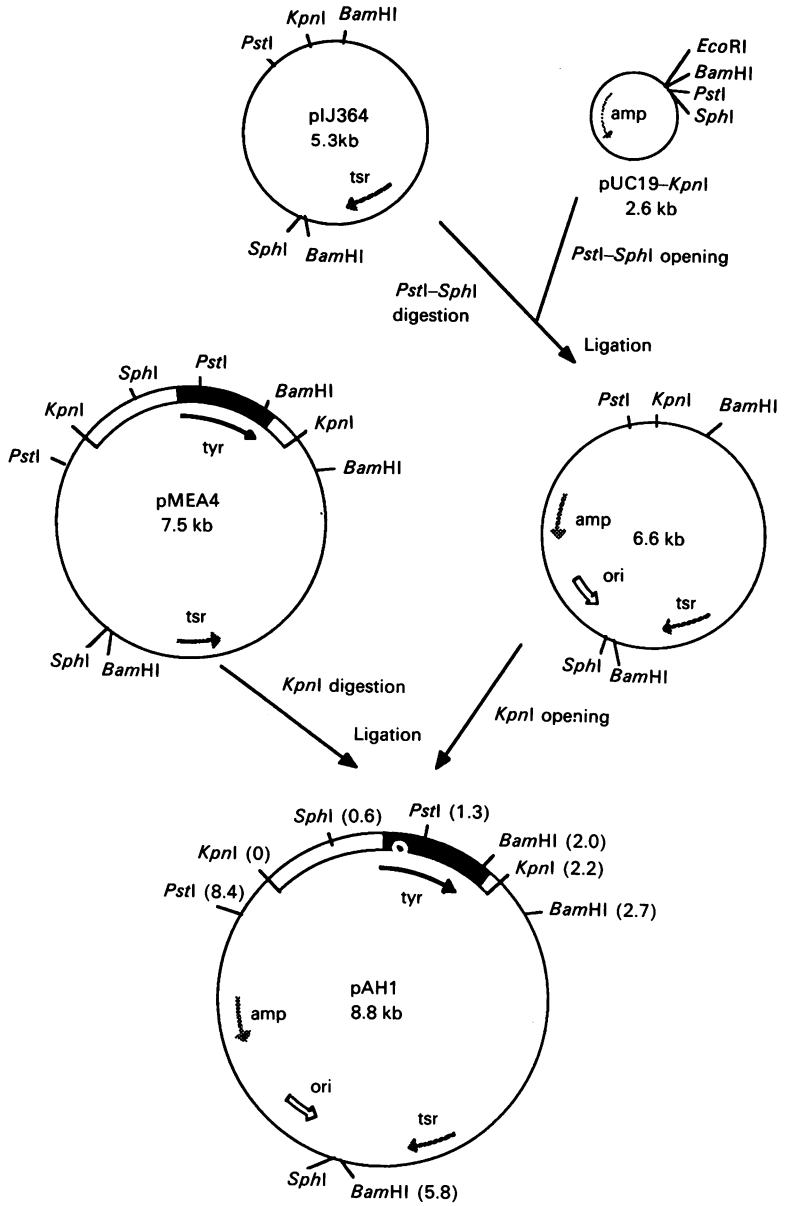

Fig. 1. Construction of the *E. coli*/*Streptomyces* shuttle vector pAH1

The vectors pIJ364 and pMEA4 have been described elsewhere (Kieser *et al.*, 1982; Hintermann *et al.*, 1985), and pUC–*Kpn*I is a derivative of pUC19 (Yanisch-Perron *et al.*, 1985) in which the *Kpn*I site was removed by *Kpn*I digestion, filling of cohesive ends with Klenow polymerase and religation. The position of ampicillin (amp)- and thiostrepton (tsr)-resistance genes and the tyrosinase open-reading frame (tyr) are indicated.

with Klenow polymerase before religating. The construction of pAH1, a vector propagated in both *E. coli* and *Streptomyces*, is shown diagrammatically in Fig. 1.

Site-directed mutagenesis

The method of Kunkel (1985) was followed. Uracil-containing templates for mutagenesis of putative Cu_A- or Cu_B-binding regions consisted of either the 700 bp *Sph*I–*Pst*I or the 480 bp *Pst*I–*Bam*HI fragment of the *S. glaucescens* tyrosinase gene (Huber *et al.*, 1985) cloned into M13mp19. Mutagenic primers were prepared by solid-phase synthesis and purified as previously described (Huber & Lerch, 1988). Screening for positive transformants by dot-blot hybridization and confirmation of the mutation by chain-termination DNA sequencing followed standard procedures (Zoller & Smith, 1983; Sanger *et al.*, 1979).

Expression of mutant tyrosinase genes using pAH1

The 700 bp or 480 bp tyrosinase gene fragment containing the point mutation was excised and ligated with *Sph*I–*Pst*I- or *Pst*I–*Bam*HI-opened pKL7 respectively. After amplification, the 2.2 kbp *Kpn*I fragment containing the complete tyrosinase gene was excised and inserted into *Kpn*I-opened pAH1. This plasmid was amplified and purified on a Quiagen tip-5 (Diagen) and used to transform *S. lividans*. Transformation, determination of phenotype on melanin-indicator plates (Hintermann *et al.*, 1985), tyrosinase expression and induction were all performed as previously described (Huber & Lerch, 1988).

Tyrosinase purification

All steps were carried out at 4 °C. Mycelia (20 g) were suspended in 100 ml of 50 mM-sodium phosphate buffer, pH 7.5,

containing 1 mM-phenylmethanesulphonyl fluoride. After sonication for 10 min in 1 s pulses and centrifugation at 8000 *g* for 5 min, the pellet was resuspended in 100 ml of buffer and resonicated. The combined extracts were centrifuged at 8000 *g* for 30 min to remove cell debris, then $CuCl_2$ was added to the supernatant to a final concentration of 10 μM. L-Dopa oxidase activity of the crude extracts was determined before and 10 min after Cu(II) addition using the dopachrome assay (Fling *et al.*, 1963). Maintenance of this Cu(II) concentration throughout chromatography was found to suppress loss of Cu from the otherwise rather labile active site.

The crude extract was passed through a Sephadex G-25 gel-filtration column (80 cm × 5 cm) equilibrated in buffer A (10 mM-Tris/HCl buffer, pH 8.6, containing 10 μM-$CuCl_2$). Tyrosinase-containing fractions were pooled and applied to a DEAE-cellulose anion-exchange column (10 cm × 4 cm) pre-equilibrated in buffer A. The enzyme was eluted with the same buffer containing 0.1 M-NaCl. The tyrosinase peak was passed through a Sephacryl S-200 gel-filtration column (80 cm × 2 cm) in buffer A. The final purification step used f.p.l.c. (Fig. 2). Protein was either desalted and used immediately or stored in 40% glycerol at −20 °C. Desalting and removal of extraneous Cu(II) was performed by passing the protein sample through a Sephadex G-25 column equilibrated in Cu-free buffer A.

Analytical and spectroscopic methods

Protein samples were analysed by electrophoresis on SDS/12.5%-polyacrylamide gels (Laemmli, 1970) stained with Coomassie Blue G250. Cu concentrations were measured using the Cu(I) chelator bathocuproinedisulphonic acid (Skotland & Ljones, 1979). Measurements were confined to the range 0.4–10 μg of Cu/ml, within the linear region of a calibration curve constructed with standard dilutions of $CuCl_2$ (Titrisol; Merck) in Cu-free buffer A. Absorption spectra were measured at 4 °C on a Kontron Uvicon 860 spectrometer. A slight molar excess of H_2O_2 was added to the protein immediately before scanning. Luminescence spectra were recorded on a Spex Fluorolog instrument at room temperature, with an excitation wavelength of 290 nm and a 450 nm emission cut-off filter. Carbonyl derivatives were prepared by placing the approx. 2 μM protein sample (in buffer A) in a 50 ml bulb connected directly to a fluorescence cuvette and equipped with a side arm containing a stopcock. The samples were reduced with a 2–3-fold molar excess of hydroxylamine, evacuated at approx. 1.3 kPa (10 Torr) for 1–2 min with gentle swirling, then the flask was refilled with CO. This procedure was repeated at least five times before inverting the cuvette and inserting directly into the fluorimeter. To determine the absorption coefficient for native tyrosinase the method of Edelhoch (1967) was followed, with freshly purified native enzyme and guanidinium chloride from Pierce Chemical Co. (Sequanal grade). The molar absorption coefficient ϵ_{280} for the mutant proteins was assumed to have the same value as that for native tyrosinase, since the amino acid exchanges do not involve tyrosine or tryptophan residues.

RESULTS

Expression of tyrosinase using pAH1

Shuttle vector pAH1, whose construction is illustrated in Fig. 1, confers both ampicillin- and thiostrepton-resistance and incorporates the essential region for replication and stability of plasmids in *Streptomyces* (Kieser *et al.*, 1982). All cloning steps could thus be performed in *E. coli*, allowing subsequent direct transformation of *Streptomyces* protoplasts by large amounts of circularly closed plasmid.

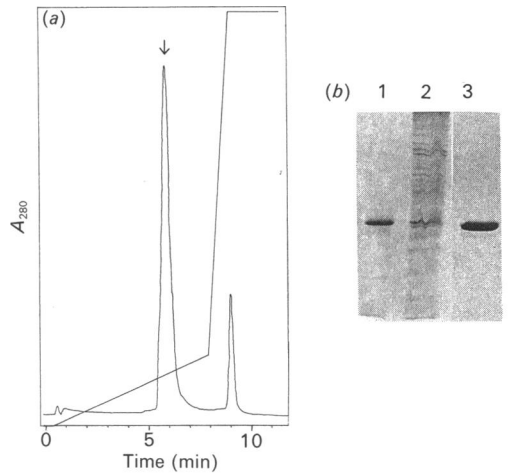

Fig. 2. Purification of *S. glaucescens* tyrosinase

(*a*) F.p.l.c. Mono-Q elution profile of *S. glaucescens* tyrosinase purified from *S. lividans* mycelial extract as described in the text. The column was pre-equilibrated in 10 mM-Tris/HCl buffer, pH 8.6. The tyrosinase peak (indicated by an arrow) was eluted by a linear gradient (1 ml/min) of 1 M-NaCl in the above buffer. (*b*) SDS/PAGE (12.5% polyacrylamide) of *S. glaucescens* tyrosinase: lane 1, purified from *S. glaucescens*; lane 2, crude *S. lividans* mycelial extract; lane 3, purified from *S. lividans* as described in the text.

The transformation efficiency of *E. coli*-derived pAH1 in *S. lividans* was 2×10^3–6×10^3 colonies/μg of DNA. On melanin-indicator plates, transformants of pAH1 containing the native *S. glaucescens* tyrosinase gene produced exclusively black colonies (phenotypically active), demonstrating the stability of pAH1 in *S. lividans*. Plasmid DNA isolated from such cultures could be retransformed efficiently in *E. coli* (results not shown).

The expression level of native tyrosinase in induced *S. lividans* liquid cultures was 1600–2000 units/g of mycelia, comparable with that obtained by homologous expression in *S. glaucescens* (Huber, 1988). However, *S. lividans* was the preferred host, owing to faster growth and sporulation, coupled with a 5-fold higher yield of mycelia (around 20 g/l) than was obtained with *S. glaucescens*.

Properties of *S. glaucescens* tyrosinase expressed in *S. lividans*

Fig. 2 illustrates the homogeneous nature of *S. glaucescens* tyrosinase after expression in *S. lividans* and purification as described in the Materials and methods section. The enzyme was indistinguishable (Cu content, u.v.–visible-absorption spectrum of the oxy-enzyme, luminescence $\lambda_{max.}$ of the carbonyl derivative) from that expressed directly in *S. glaucescens*. It was necessary to redetermine the absorption coefficient for *S. glaucescens* tyrosinase, since an earlier estimate (Huber & Lerch, 1988) was erroneously based on an amino acid composition with underestimated tyrosine and tryptophan content (Lerch & Ettlinger, 1972). In the present determination, the ϵ_{280} calculated for the enzyme's 12 tryptophan and eight tyrosine residues (Huber *et al.*, 1985) was corrected by the factor 1.05 to allow for observed absorbance differences between folded and unfolded forms (Edelhoch, 1967) yielding ϵ_{280} $8.21 \times 10^4 \pm 0.13 \times 10^4$ $M^{-1} \cdot cm^{-1}$.

Design and isolation of mutant enzymes

Tyrosinases and haemocyanins share a wealth of spectroscopic properties (Wilcox *et al.*, 1985; Kuiper *et al.*, 1980*a*,*b*), which are assumed to reflect common structural features of their binuclear

Cu_B region

```
                          NQ  Q                    Q
                          ↓↓  ↓                    ↓
S. glaucescens  (189)   HNRVH  - (208) - DPVFWLHH
pmcTyr1         (345)   HNALH  - (365) - DPIFLLHH
Pmel34          (350)   HNALH  - (369) - DPIFLLHH
N. crassa       (277)   HNEIH  - (299) - DPLFLLHH
H. pomatia d    (175)   HNALH  - (199) - DPVFFLHH
P. interruptus  (351)   HNTAH  - (384) - DPSFFRLH
                        *   *                *
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Cu_A region

```
                                Q              Q        N
                                ↓              ↓        ↓
S. glaucescens   (30)   YDEFVTTHNA  -  (51) - TGHRSPSFLPWHR
pmcTyr1         (155)   YDLFVWMHYY  - (182) - FAHEAPGFLPWHR
Pmel34          (160)   YDLFVWMHYY  - (187) - FAHEAPAFLPWHR
N. crassa               - - - - - -  - (93) - CTHSSILFITWHR
H. pomatia d     (37)   YENIASFHGK  -  (53) - EGHKMPTFPSWHR
                                                  ^
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Fig. 3. Amino acid sequence alignment between putative Cu_A- and Cu_B-binding regions of *S. glaucescens* tyrosinase and those of related proteins

Source of sequences: pmcTyr1 (*Mus musculus* tyrosinase, Müller *et al.*, 1988); Pmel34 (*Homo sapiens* tyrosinase, Kwon *et al.*, 1987); *N. crassa* tyrosinase (Lerch, 1978); *Helix pomatia* haemocyanin subunit d (Drexel *et al.*, 1986); *P. interruptus* haemocyanin (Linzen *et al.*, 1985). The point mutations described are indicated above the *S. glaucescens* sequence. The *P. interruptus* Cu_B ligands are asterisked. An insertion of seven amino acid residues in the *H. pomatia* sequence is indicted by ^.

H37Q

```
5'- CC ACC CAC AAC GCC TTC -3'
3'- GG TGG GTC TTG CGG AAG -3'
          *
    -Thr-Gln-Asn-Ala-Phe-
```

H53Q

```
5'- C ACC GGC CAC CGC TCG C -3'
3'- G TGG CCG GTC GCG AGC G -5'
          *
    -Thr-Gly-Gln-Arg-Ser-
```

H193Q

```
5'- C CGC GTC CAC GTC TGG G -3'
3'- G GCG CAG GTT CAG ACC C -5'
          *
    -Arg-Val-Gln-Val-Trp-
```

H215Q

```
5'- G CTG CAC CAC GCC TAC G -3'
3'- C GAC GTG GTC CGG ATG C -5'
          *
    -Leu-His-Gln-Ala-Tyr-
```

N190Q

```
5'- AC CTG CAC AAC CGC GTC C -3'
3'- TG GAC GAG GTC GCG CAG G -5'
          * *
    -Leu-His-Gln-Arg-Val-
```

Fig. 4. Design of oligonucleotide primers for site-directed mutagenesis of the *S. glaucescens* tyrosinase gene

In each case the coding (template) strand of the wild-type sequence is shown above the oligonucleotide primer, and the mutant protein sequence below. Point mutations are indicated by ★.

copper active sites (Lerch, 1988). Present knowledge of the three-dimensional structures of these proteins is limited to that of haemocyanin from the arthropod *P. interruptus* (Gaykema *et al.*, 1984), which reveals that each Cu atom is co-ordinated to three histidine residues in a characteristic fashion. Amino acid sequence comparison in the highly conserved region containing the Cu_B ligands of *P. interruptus* haemocyanin (Fig. 3) shows that these three histidine residues are invariant in all tyrosinases and haemocyanins yet sequenced (Linzen *et al.*, 1985; Müller *et al.*, 1988). In *S. glaucescens* tyrosinase, the first of these histidine residues, His-189, has been implicated, by using site-directed mutagenesis, as a Cu ligand (Huber & Lerch, 1988). As part of the present study, we have replaced the two remaining invariant histidine residues in the Cu_B region by non-co-ordinating glutamine (mutants H193Q and H215Q). The only other *S. glaucescens* histidine residue lying within the Cu_B region, His-214, is not present in the *P. interruptus* sequence. In addition, experiments with *N. crassa* tyrosinase showed that the analogue of *S. glaucescens* residue His-215 (and not His-214) was specifically destroyed by reaction-inactivation of the active site (Dietler & Lerch, 1982).

The Cu_A-binding region of *P. interruptus* haemocyanin lies further towards the *N*-terminus and shows limited sequence similarity to *N. crassa* tyrosinase (Lerch, 1978). However, molluscan haemocyanins (Drexel *et al.*, 1986), *Streptomyces* and mammalian tyrosinases (Huber *et al.*, 1985; Kwon *et al.*, 1987; Müller *et al.*, 1988) have no sequence similarity to this region. Instead, they share conserved sequences (Fig. 3) incorporating three out of four histidine residues lying, at positions 37, 53 and 62, on the *N*-terminal side of the proposed Cu_B site in *S. glaucescens* tyrosinase (Huber & Lerch, 1985). The region containing the fourth histidine residue, His-180, has no sequence

Table 1. Properties of the purified tyrosinase mutants

Values in parentheses were measured in the presence of extraneous 10 μM-Cu(II).

	Cu content (mol/mol of enzyme)	Dopa oxidase activity (units/mg)	Luminescence of carbonyl derivative	
			$\lambda_{max.}$ (nm)	Intensity (% of native intensity)
Native	(1.9±0.2) 1.8±0.1	2500	538	100
H37Q	(1.7±0.2) 1.1±0.1	< 0.2	–	< 0.1
H53Q	(1.8±0.2) 0.9±0.1	< 0.2	–	< 0.1
H62N	(1.6±0.3) 1.1±0.1	< 0.2	–	< 0.1
H189N	(2.5±0.2) 1.8±0.1	< 0.2	572	0.5
H193Q	(1.4±0.2) 1.0±0.1	0.4	565	15
H215Q	(2.1±0.3) 1.7±0.1	0.9	–	< 0.1
N190Q	(1.6±0.2) 0.9±0.1	1.3	545 (540)	5 (20)

similarity to haemocyanin or mammalian tyrosinase sequences. Following the recent implication of His-62 as a Cu ligand in *S. glaucescens* (Huber & Lerch, 1988), we wished to identify other potential Cu_A ligands of *S. glaucescens* tyrosinase, and to this end His-37 and His-53 were replaced by glutamine (mutants H37Q and H53Q respectively). The remaining three histidine residues in the *S. glaucescens* sequence (*C*-terminal of the proposed Cu_B site at positions 230, 264 and 267) cannot be aligned with mammalian tyrosinase sequences to produce a putative Cu-binding region, and were not investigated by mutagenesis.

An additional mutant was prepared to test the importance of an asparagine residue found at the active site of *P. interruptus* haemocyanin (Gaykema *et al.*, 1984) and invariant in all tyrosinases and haemocyanins. Asp-190 of *S. glaucescens* tyrosinase was replaced by glutamine (mutant N190Q). Design of mutagenic primers and the concomitant point mutations are shown in Fig. 4. Cloning of the mutant genes into pAH1 was followed by transformation, expression and purification as for native tyrosinase. In order to complete the comparison of proposed histidine ligands, mutants H62N and H189N, which had been expressed in *S. glaucescens*, were also purified as described in the Materials and methods section.

Properties of the mutant proteins

All mutants described here yielded exclusively white colonies on melanin-indicator plates (Hintermann *et al.*, 1985). A similar result has been observed for mutants H62N and H189N expressed in *S. glaucescens* (Huber & Lerch, 1988). Consistent with their albino phenotypes, L-dopa oxidase activity in crude extracts of the mutant proteins was less than 0.05 units/g of cells, compared with 1600 units/g in the native extract. The activity redetermined 10 min after $CuCl_2$ addition had risen to as high as 0.4 unit/g for mutant and 1870 units/g for native extracts.

Mutant tyrosinases were purified to greater than 95% homogeneity as judged by SDS/PAGE (Fig. 2). The Cu content of these proteins is presented in Table 1. Values measured before removal of extraneous Cu(II) were corrected to allow for the presence of 10 μM background Cu(II). Since the tyrosinase concentration always exceeded 10 μM in these measurements, the correction did not introduce large errors into the determined values. For example, the measured values for N193Q were: Cu, 28.0±0.2 μM (corrected, 18.0±0.3 μM); tyrosinase, 13.0±0.1 μM; ratio, 1.4±0.2. The Cu content thus obtained was markedly higher than that observed after gel filtration into Cu-free buffer (Table 1). In contrast, the Cu content of native tyrosinase remained essentially unchanged during this process. The specific activity of the purified enzymes, also presented in Table 1, did not increase in the presence of extraneous Cu(II). The absorption spectra of these mutants all lack the $O_2^{2-} \rightarrow Cu(II)$ charge-transfer band characteristic of oxy-tyrosinase (Solomon, 1981).

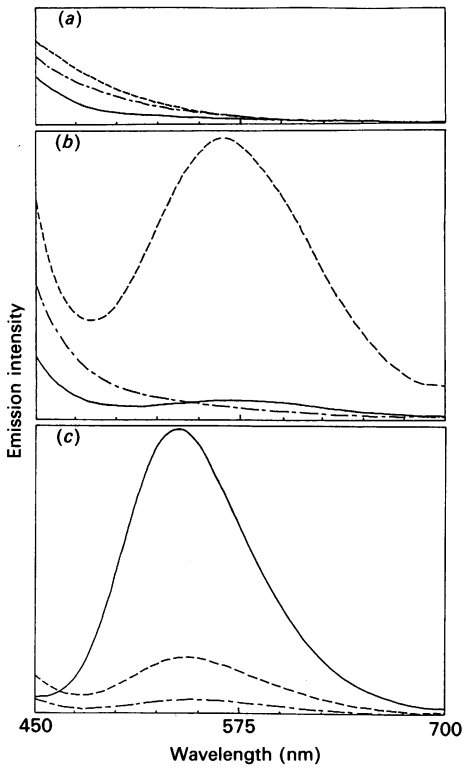

Fig. 5. Luminescence spectra of carbonyl tyrosinase

(*a*) Cu_A histidine mutants H37Q (——), H53Q (– – –) and H62N (-·-·-). (*b*) Cu_B histidine mutants H189N (——), H193Q (– – –) and H215Q (-·-·-). (*c*) Native enzyme (——), mutant N190Q in the presence (– – –) or in the absence (-·-·-) of extraneous 10 μM-Cu(II). The intensity scale is reduced 6.5-fold compared with (*a*) and (*b*).

The luminescence spectra were found to be strongly dependent on the nature of the mutation. No luminescence peak above 450 nm could be detected for CO-saturated samples of the Cu_A histidine mutants or for H215Q (Figs. 5*a* and 5*b*), despite a detection limit of around one-thousandth the native intensity. However, for mutants H189N and H193Q, luminescence was

observed. Reduced luminescence intensity of the carbonyl derivatives was coupled with a striking shift in $\lambda_{max.}$ of more than 25 nm to a longer wavelength (Fig. 5*b*). The luminescence $\lambda_{max.}$ of carbonyl-N190Q, in contrast, was shifted only a few nanometres from that of the native enzyme (Fig. 5*c*). Remarkably, if 10 μM-$CuCl_2$ was added and the sample resaturated with CO, the $\lambda_{max.}$ and intensity resembled those of native tyrosinase. In contrast, no effect of Cu(II) concentration was observed for the histidine mutants.

DISCUSSION

The *E. coli*/*Streptomyces* shuttle vector pAH1 has been used to express the gene for *S. glaucescens* tyrosinase in a tyrosinase-deficient strain of *S. lividans*. This system provides an enhanced level of expression of native tyrosinase (up to 40000 units/l of culture), exhibiting the same properties as the homologously expressed enzyme (Huber & Lerch, 1988).

S. glaucescens tyrosinase is notably more thermolabile than the enzymes from *N. crassa* and *A. bisporus* (Lerch, 1983; Schoot Uiterkamp & Mason, 1973). Indeed, despite a molecular mass of 30 kDa, it contains no disulphide bridges. This is reflected in the lability of the active-site Cu complex, and early preparations of the enzyme were found to contain only 1 Cu atom/molecule (Lerch & Ettlinger, 1972). Interestingly, we find that the specific activity can be slightly increased by adding 10 μM-$CuCl_2$ to crude enzyme extracts, suggesting that not all active sites are fully occupied in the enzyme population *in vivo*. Cu lability is even more extreme in the mutants described here. This prompted us to purify the proteins in the presence of 10 μM-Cu(II) in order to suppress active-site Cu loss. In further mutants of *S. glaucescens* tyrosinase, not described in the present paper, stabilization of the $Cu(II)_2$ oxy-enzyme has been observed (K. Lerch, M. Huber, A. Hajnal & M. P. Jackman, unpublished work).

Histidine mutants

The amino acid sequence alignment in Fig. 3 implicates His-37, -53, -62, -189, -193 and -215 as potential Cu ligands at the binuclear active site of *S. glaucescens* tyrosinase. The participation of His-62 and His-189 was confirmed through their mutation to asparagine (Huber & Lerch, 1988), and here we describe the effect of exchanging His-37, -53, -193 and -215 with glutamine. The following evidence supports their involvement as Cu ligands: (i) the albino phenotype and absence of an $O_2^{2-} \rightarrow Cu(II)$ charge-transfer band in the glutamine mutants indicates substantial perturbation of the binuclear active site; (ii) the loss of approximately 1 Cu atom/molecule upon removal of extraneous Cu(II) is consistent with weaker Cu co-ordination following exchange of an active-site histidine for a non-co-ordinating residue.

The Cu content of H189N and H215Q after purification is somewhat higher than that of the other mutants. This anomaly may possibly have arisen from specific binding of extraneous Cu(II) to these mutants during purification, since H189N prepared in the absence of extraneous Cu(II) yielded a Cu content of 0.95 (Huber & Lerch, 1988). Another possibility is that these mutations may have increased ϵ_{280}, resulting in underestimation of the protein concentration. In any case, their Cu lability relative to the native enzyme has increased.

The results suggest that three Cu_A and three Cu_B histidine ligands are required in *S. glaucescens* tyrosinase, as observed also in the X-ray crystal structure of *P. interruptus* deoxy-haemocyanin (Gaykema *et al.*, 1984). In this structure, each Cu(I) is seen to bind two equatorial histidine residues strongly and co-ordinate a third axial histidine residue weakly. If this geometry were conserved in *S. glaucescens* tyrosinase, then by sequence analogy with the Cu_B region of haemocyanins (Fig. 3) His-193 would be an axial ligand. The available data do not permit an axial Cu_A ligand of tyrosinase to be distinguished. Models of the various oxidation states of the binuclear Cu complex, based on spectroscopic evidence (Solomon, 1981), incorporate only the four equatorially co-ordinating histidine residues. This is also the case for the proposed tyrosinase reaction mechanism (Wilcox *et al.*, 1985; Lerch, 1988). The albino phenotype of all six histidine mutants described here raises the possibility of a more important role for the axial ligands than previously expected.

The apparent involvement of His-37 as a Cu ligand further emphasizes the structural diversity of the Cu_A-binding regions of *S. glaucescens* and *N. crassa* tyrosinases and arthropodan haemocyanins appears somewhat sensitive to the active-site that amino acid sequences of mammalian tyrosinases (and a molluscan haemocyanin) share conserved regions comprising all six of the proposed *S. glaucescens* Cu ligands (Fig. 3). *S. glaucescens* tyrosinase may thus be considered a good model for the related mammalian enzymes, which have so far eluded spectroscopic characterization.

It has long been established that haemocyanin binds one CO molecule per active centre in a terminal non-bridging geometry (Yen Fager & Alben, 1972), and this is also assumed to hold for tyrosinases (Kuiper *et al.*, 1980*b*). Substitution of the putative Cu_A-binding histidine residues by either asparagine or glutamine abolishes luminescence, whereas for the Cu_B mutants only H215Q failed to luminesce. If, as seems reasonable to assume, Cu is labilized at the site of histidine mutation, this would suggest that CO binds to Cu_A of *S. glaucescens* tyrosinase. Since the relative Cu occupancy of the Cu_A and Cu_B sites in H193Q was not determined, we cannot exclude the possibility that a small population of intact binuclear sites is responsible for the observed luminescence. However, the independence of luminescence intensity on extraneous Cu concentration argues against this.

The luminescence $\lambda_{max.}$ of carbonyl-tyrosinases and haemocyanins appears somewhat sensitive to the active-site environment. Peak positions between 537 and 560 nm have been reported (Finazzi-Agrò *et al.*, 1982; Sorrell *et al.*, 1988), with arthropodan haemocyanins tending to exhibit maxima at the higher end of the range. The magnitude of the spectral peak shift for carbonyl-H189N and -H193Q further emphasizes the strong active-site perturbation in these mutants. The specific factors involved in tuning the luminescence peak position remain, however, unresolved.

Mutant N190Q

The role of an invariant asparagine residue in the Cu_B-binding region of tyrosinases and haemocyanins (Linzen *et al.*, 1985; Müller *et al.*, 1988) was examined by preparing *S. glaucescens* mutant N190Q. The effective introduction of a $-CH_2-$ group without changing the functional nature of the residue can be regarded as a very conservative substitution (Knowles, 1987). Surprisingly, the L-dopa oxidase activity of N190Q is around 2000-fold lower than for the native enzyme, and, together with the absence of a detectable oxy-enzyme, it shows that perturbation of the active site has occurred. Unlike the histidine mutants, however, luminescence of carbonyl-N190Q resembles that of native tyrosinase, particularly at Cu concentrations promoting double occupancy of the active site. Examination of the *P. interruptus* haemocyanin structure (Gaykema *et al.*, 1984) reveals that the corresponding residue, Asn-352, is involved in an active-site hydrogen-bonding network that bridges the two Cu atoms. It would appear that the introduction of a $-CH_2-$ group

in mutant N190Q does not lead to substantial active-site conformational changes, but is sufficient to disturb the delicate network of hydrogen-bonding interactions essential for enzyme activity, leading to labilization and loss of one Cu atom.

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