Malin, E. L., Greenberg, R., & Farrell, H. M., Jr. (1979) *Abstracts of the XIth International Congress of Biochemistry*, p 185, National Research Council of Canada, Ottawa.
Mattarella, N. L., Creamer, L. K., & Richardson, T. (1983) *J. Agric. Food Chem. 31*, 968–972.
McKenzie, H. A., & Sawyer, W. H. (1972) *Aust. J. Biol. Sci. 25*, 949–961.
Means, G. E., & Feeney, R. E. (1968) *Biochemistry 7*, 2192–2201.
Meek, J. L. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1632–1636.
Meek, J. L., & Rossetti, Z. L. (1981) *J. Chromatogr. 211*, 15–28.
Meites, L. (1979) *CRC Crit. Rev. Anal. Chem. 8*, 1–53.
Newcomer, M. E., Jones, T. A., Aqvist, J., Sundelin, J., Eriksson, U., Rask, L., & Peterson, P. A. (1984) *EMBO J. 3*, 1451–1454.
Pace, C. N., & Marshall, H. F., Jr. (1980) *Arch. Biochem. Biophys. 199*, 270–276.
Papiz, M. Z., Sawyer, L., Eliopoulos, E. E., North, A. C. T., Findlay, J. B. C., Sivaprasadarao, R., Jones, T. A., Newcomer, M. E., & Kraulis, P. J. (1986) *Nature (London) 324*, 383–385.
Pervaiz, S., & Brew, K. (1985) *Science (Washington, D.C.) 222*, 335–337.
Poncz, L., & Dearborn, D. G. (1983) *J. Biol. Chem. 258*, 1844–1850.
Preaux, G., Braunitzer, G., Schrank, B., & Stangl, A. (1979) *Hoppe-Seyler's Z. Physiol. Chem. 360*, 1595–1604.
Rowley, B. O., Lund, D. B., & Richardson, T. (1979) *J. Dairy Sci. 62*, 533–536.
Sawyer, L., Papiz, M. Z., North, A. C. T., & Eliopoulos, S. E. (1985) *Biochem. Soc. Trans. 13*, 265–266.
Schechter, Y., Pathchornik, A., & Burstein, Y. (1973) *Biochemistry 12*, 3407–3413.
Segrest, J. P., & Feldman, R. J. (1977) *Biopolymers 16*, 2053–2065.
Sherry, A. D., & Teherani, J. (1983) *J. Biol. Chem. 258*, 8663–8669.
Smith, I. C. P. (1979) *Can. J. Biochem. 57*, 1–14.
Smith, L. M., Fantozzi, P., & Creveling, R. K. (1983) *J. Am. Oil Chem. Soc. 60*, 960–967.
Timasheff, S. N., & Townend, R. T. (1969) in *Protides of the Biological Fluids*, pp 33–40, Pergamon, New York.
Townend, R., & Timasheff, S. N. (1960) *J. Am. Chem. Soc. 82*, 3168–3174.
Townend, R., Kumosinski, T. F., & Timasheff, S. N. (1967) *J. Biol. Chem. 242*, 4538–4545.
Vold, R. L., Waugh, J. S., Klein, M. P., & Phelps, D. E. (1968) *J. Chem. Phys. 48*, 3831–3832.
Weiner, A. M., Platt, T., & Weber, K. (1972) *J. Biol. Chem. 247*, 3242–3251.
Wishnia, A., & Pinder, T. W., Jr. (1966) *Biochemistry 5*, 1534–1542.

Identification of Two Histidines as Copper Ligands in *Streptomyces glaucescens* Tyrosinase†

Marcel Huber and Konrad Lerch*
Biochemisches Institut der Universität Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland
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ABSTRACT: The physicochemical properties of wild type and two mutants of *Streptomyces glaucescens* tyrosinase are reported. The native enzyme contains two coppers at the active site which are EPR nondetectable. The two coppers react stoichiometrically with one hydrogen peroxide molecule giving rise to oxytyrosinase. Its optical features are similar to those reported earlier for a molluscan hemocyanin. The two mutants in which histidine-62 and -189 were changed to asparagine by site-directed mutagenesis have lost their enzymatic activity and their ability to bind oxygen and contain only one copper ion which is fully EPR detectable. The EPR parameters indicate that the remaining copper is in a tetragonally distorted ligand environment. These data are in agreement with His-62 and His-189 serving as copper ligands in *S. glaucescens* tyrosinase.

The copper-containing monooxygenase tyrosinase (EC 1.14.18.1) catalyzes both the orthohydroxylation of monophenols and the oxidation of *o*-diphenols to *o*-quinones (Mason, 1965). This enzyme is widely distributed in nature where it is responsible for the formation of melanin pigments (Lerch, 1981). During the last few years the enzymes of *Neurospora crassa* and *Agaricus bisporus* have been studied most extensively both from a structural and from a functional point of view. They were shown to contain an antiferromagnetically coupled copper pair at their active site (Schoot Uiterkamp & Mason, 1973; Lerch, 1983) with spectroscopic properties very similar to those reported for hemocyanins (Solomon, 1981). This similarity is also borne out by a comparison of the primary structures of tyrosinases and hemocyanins. All proteins sequenced so far contain a highly similar region, designated as Cu(B), with three conserved histidyl residues (Huber et al., 1985; Lerch et al., 1986) which were shown to be copper ligands in *Panulirus interruptus* hemocyanin by X-ray crystallography (Gaykema et al., 1984). Concerning the Cu(A) site (Gaykema et al., 1984), only partial sequence identity exists between *N. crassa* tyrosinase and arthropodan hemocyanins (Lerch et al., 1986). No region similar to these Cu(A) sites was found in the tyrosinases from *Streptomyces glaucescens*, a melanin-producing Gram-positive bacteria (Huber et al., 1985), and mouse (Shibahara et al., 1986). However,

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N. crassa and *S. glaucescens* tyrosinases share another similar region containing two conserved histidines (Huber et al., 1985). To test the hypothesis that this region in the bacterial enzyme might be involved in copper binding, histidine-62 (Huber et al., 1985) was replaced with asparagine by site-directed mutagenesis. Furthermore, histidine-189 (Huber et al., 1985) was changed to asparagine as well to confirm its function as a ligand to Cu(B).

The physicochemical properties of the two mutants are reported and compared to those of the native enzyme. Both mutants have lost their enzymatic activity and were found to bind only one copper contrary to the wild-type enzyme which contains a binuclear copper center.

Materials and Methods

Bacterial Strains and Plasmids. The following strains were used for the propagation of M13 phages: W71.18 (Δlacpro, rpsl/F'lacI^q, lacZ M15, pro A$^+$B$^+$), CJ236 [dut1, ung1, thi1l, relA1/pCJ105(Cm^r)], and CSH50 (Δprolac, thi, ara, strA/F'proA$^+$B$^+$, lacI^q, lacZ M15, tra D36). They were grown either on M9 minimal plates (Miller, 1972) or 2xYT plates (Miller, 1972) supplemented with chloramphenicol (20 μg/mL) in the case of CJ236 (Joyce & Grindley, 1984). As host for *Streptomyces* plasmids, the strain GLA 212 (melC-212, strS-212, ETH Zürich) was used. It was kept as a spore suspension in 20% (v/v) glycerol at −20 °C.

The M13 phage vectors used for the cloning and mutagenesis experiments were M13mp18 and M13mp19 (Yanisch-Peron et al., 1985). The *Streptomyces* plasmids pMEA4 and pIJ364 have been described earlier (Hintermann et al., 1985; Kieser et al., 1982).

Oligonucleotide Synthesis. The mutagenic primers KL-9 (5'-CTGGAACCGCAGATA-3') and KL-11 (5'-CCTGAACAACCGCGT-3') were synthesized with a manual solid-phase synthesizer (Bachemgentech) using the phosphotriester method (Miyoshi et al., 1980a,b). The oligonucleotides were further purified by electrophoresis on a denaturing polyacrylamide gel (20%) and ion-exchange chromatography on NACS-52 (BRL).

Site-Directed Mutagenesis. The mutation His-62 → Asn (N62) was introduced with KL-9 essentially as described by Zoller and Smith (1983) with the following modifications: M13 universal sequencing primer was included in the annealing step, and the alkaline sucrose gradient was omitted. The *Kpn*I–*Kpn*I fragment of pMEA4 (Hintermann et al., 1985) was subcloned into M13mp18, and the phage DNA, prepared as published earlier (Zoller & Smith, 1983), was used as a template. The second mutation His-189 → Asn (N189) was achieved by annealing KL-11 to an uracil-containing template, consisting of the 476 base pair long *Bam*HI–*Pst*I fragment of pMEA7 (Huber et al., 1985) cloned into M13mp18. The preparation of the uracil-containing template and the heteroduplex synthesis were performed as described (Kunkel, 1985). The heteroduplex was transformed into $CaCl_2$-treated (Mandel & Higa, 1970) CSH50 cells which select against the uracil-containing template (Kunkel, 1985). Positive clones for both mutations were identified by screening dot blots on nitrocellulose membranes with the radioactively labeled mutagenic primers (Zoller & Smith, 1983). Finally, the mutations were sequenced by the chain-termination method (Sanger et al., 1979). The fragments containing the mutations were then cloned into the *S. glaucescens* plasmid pMEA4 (Hintermann et al., 1985).

Expression of Wild-Type and Mutant Tyrosinases. Plasmid pMEA4 (wild type and mutants) was introduced into GLA212, a melC mutant (Crameri et al., 1984) lacking the structural tyrosinase gene, by protoplast transformation (Thompson et al., 1982). Transformation mixtures were spread on regeneration plates (Thompson et al., 1980), and positive clones were selected by overlaying the plates with soft nutrient agar containing thiostrepton (500 μg/L), L-tyrosine (2.5 g/L), L-leucine (1.2 g/L), L-methionine (0.25 g/L), and $CuSO_4$ (25 mg/L) (melanin indicator plates; Hintermann et al., 1985). Spore suspensions of positive transformants were used to inoculate cultures in minimal medium (Baumann & Kocher, 1976) supplemented with thiostrepton (5 μg/L). After the cultures were shaken for 48 h at 30 °C, the expression of tyrosinase was elicited by the addition of L-leucine (400 mg/L), L-methionine (100 mg/L), and $CuSO_4$ (5 mg/L). After a further 4–6 h, mycelia were harvested by filtration through a Büchner funnel and stored at −80 °C.

Purification of Wild-Type and Mutant Tyrosinases. All steps were carried out at 4 °C. Mycelia were disrupted by sonification (Branson sonifier) for 10 min in 50 mM sodium phosphate, pH 7.5, supplemented with 1 mM phenylmethanesulfonyl fluoride. After centrifugation, the supernatant was applied to a G-25 column equilibrated in 10 mM Tris-HCl, pH 8.6. Fractions containing tyrosinase activity were pooled, and the enzyme was purified by DEAE-cellulose[1] chromatography in 10 mM Tris-HCl, pH 8.6, and eluted with 0.1 M sodium chloride in the same buffer. Subsequently, the fractions containing tyrosinase activity were applied on a CM-Sephadex column in 10 mM sodium phosphate, pH 6, and the enzyme was eluted with a pH gradient (10 mM sodium phosphate, pH 6, to 100 mM sodium phosphate, pH 8). The enzyme was concentrated with a small DEAE-cellulose column (1 × 4 cm) as described above. Purified enzyme was stored in 10 mM Tris-HCl, pH 8.6/0.1 M sodium chloride/40% (v/v) glycerol at −20 °C. Tyrosinase activity was determined by the dopachrome assay (Fling et al., 1963). Protein concentrations were measured either according to Lowry et al. (1951) or with the extinction coefficient A_{280} = 19 (10 mg/mL) for the purified enzyme.

Preparation of Mettyrosinase. The met derivative of *S. glaucescens* tyrosinase was prepared by the addition of a 5-fold molar excess of L-mimosine. The sample was loaded afterward on a G-25 column (0.8 × 29 cm) in 50 mM sodium phosphate, pH 7.2, to remove excess L-mimosine.

Gel Electrophoresis. Samples of crude extracts and purified enzyme were analyzed on a 10% SDS–polyacrylamide gel in a discontinuous buffer system (Laemmli, 1970) and stained with Coomassie Blue G250.

Spectroscopic Measurements. Absorption spectra were recorded on a Uvicon 810 spectrophotometer (Kontron) at 4 °C. CD spectra were recorded at room temperature with a JASCO J500C spectropolarimeter connected to an Epson QX-10 computer. Spectra were smoothed twice before plotting. The concentration of the hydrogen peroxide stock solution was determined titrimetrically with a $KMnO_4$ standard solution (Merck). EPR spectra were measured on a Bruker Electrospin spectrometer at 77 K and 9.46 GHz. The signal of diphenylpicryl hydrazide was used to calibrate the magnetic field. EPR-detectable copper was estimated by double integration of the spectra, applying corrections for the different g factors (Aasa & Vänngard, 1975). Cu,Zn-superoxide dismutase of *N. crassa* was used as standard.

Copper Determination. Copper concentration was measured with the Cu(I) chelator bathocuproinedisulfonic acid as de-

[1] Abbreviations: CD, circular dichroism; CM, carboxymethyl; DEAE, diethylaminoethyl; EPR, electron paramagnetic resonance; SDS, sodium dodecyl sulfate.

Table I: Purification Scheme for *S. glaucescens* Tyrosinase

purification step	total protein (mg)	total act. (units)	sp act. (unit/mg)	purification (*x*-fold)	yield (%)
crude extract	272	43450	160	1	100
DEAE-cellulose chromatography	31.5	33300	1060	6.6	76.6
CM-Sephadex chromatography	12.6	26450	2100	13.1	60.9

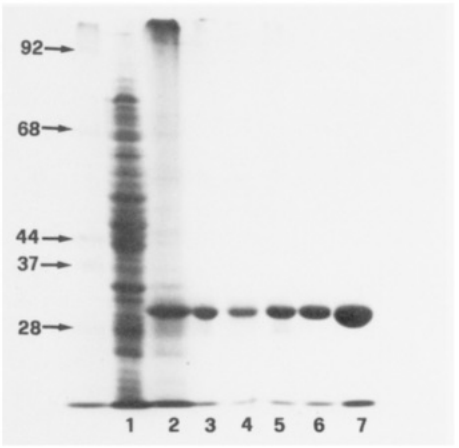

FIGURE 1: SDS–polyacrylamide gel analysis of the purification of wild-type and mutant enzymes: (lane 1) crude extract of GLA212 transformed with pIJ364; (lane 2) crude extract of GLA212 transformed with pMEA4; (lane 3) DEAE-cellulose pool; (lane 4) CM-Sephadex pool; (lane 5) purified wild-type enzyme; (lane 6) purified N62 mutant enzyme; (lane 7) purified N189 mutant enzyme. The numbers on the left give the molecular weights in kilodaltons of the marker proteins.

scribed earlier (Skotland & Ljones, 1979). The $CuSO_4$ standard solution was calibrated by atomic absorption spectroscopy.

RESULTS

Characterization of the Wild-Type Enzyme. S. glaucescens tyrosinase was isolated from the strain GLA212 transformed with the plasmid pMEA4 (Hintermann et al., 1985), a derivative of the high-copy number, broad host range vector pIJ364 (Kieser et al., 1982). This allowed the expression of tyrosinase to about 10–15% of the total protein as judged from the specific activity in a crude extract. Table I shows the purification scheme of *S. glaucescens* tyrosinase. The overall yield is 61%, which compares favorably with the one of an earlier isolation procedure (Lerch & Ettlinger, 1972). The purified enzyme yielded a single band on an SDS–polyacrylamide gel (Figure 1). Furthermore, the amino acid composition is in good agreement with the one deduced from the nucleotide sequence (Huber et al., 1985) as shown in Table II.

Upon addition of a 5-fold molar excess of hydrogen peroxide to *S. glaucescens* tyrosinase, a very prominent band at 345 nm and a weaker, broad one at 640 nm arise in the absorption spectrum (Figure 2). These spectral features are typical for the oxy form of tyrosinase (Solomon, 1981). The CD spectrum of the oxy form of *S. glaucescens* tyrosinase is shown in Figure 2.

Figure 2 (inset) shows a titration of *S. glaucescens* tyrosinase with hydrogen peroxide. The increase of the absorbance at 345 nm was constant over several additions of hydrogen peroxide indicating a stoichiometric reaction of H_2O_2 with the copper center. From the slope of the curve an extinction coefficient $\epsilon_{345} = 16.3 \times 10^3\ M^{-1}\ cm^{-1}$ was calculated. This

Table II: Amino Acid Composition of Purified *S. glaucescens* Tyrosinase

amino acid	protein[a]	DNA[b]	amino acid	protein[a]	DNA[b]
Asx	33.0	34	Met	4.4	5
Thr[c]	17.3	19	Ile	3.0	3
Ser[c]	15.4	16	Leu	21.9	23
Glx	14.6	15	Tyr[c]	7.3	8
Pro	14.3	15	Phe	9.0	10
Gly	22.6	22	His	11.4	11
Ala	29.0	29	Lys	4.7	5
Val	17.7	20	Arg	26.0	26

[a] Mean value obtained after 20-h hydrolysis (Moore & Stein, 1963). [b] Huber et al., 1985. [c] Corrected for partial loss during hydrolysis.

Table III: Extinction Coefficients of H_2O_2-Treated Tyrosinases and Reaction Stoichiometry

	ϵ_{345,H_2O_2} ($M^{-1}\ cm^{-1}$)	$\epsilon_{345,Cu}$ ($M^{-1}\ cm^{-1}$)	Cu/H_2O_2
mushroom[a]	17.2×10^3	9.1×10^3	0.53
N. crassa[b]	16.9×10^3	9.0×10^3	0.53
S. glaucescens	16.3×10^3	8.7×10^3	0.54

[a] Jolley et al., 1974. [b] Lerch, 1976.

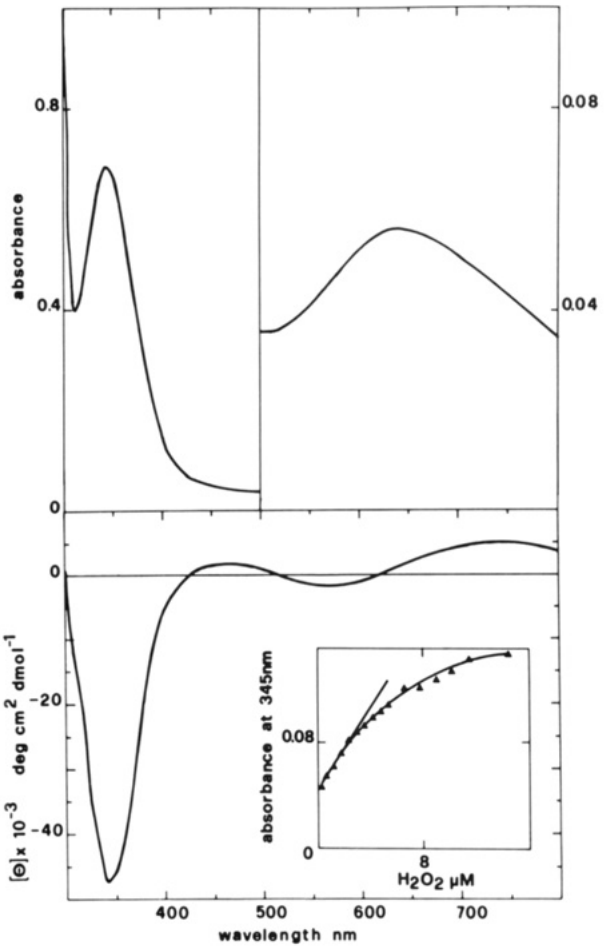

FIGURE 2: Absorption and CD spectra of wild-type oxytyrosinase (60 μM) of *S. glaucescens* (10 mM Tris-HCl, pH 8.6/0.1 M NaCl at 4 °C). The inset shows the titrimetric determination of the extinction coefficient at 345 nm of oxytyrosinase. Aliquots of 0.5 μM H_2O_2 were added to mettyrosinase (15 μM).

value compares favorably with those obtained from similar titration experiments with *N. crassa* and mushroom tyrosinases (Table III). The ratio $Cu:H_2O_2$ was found to be 0.54 (Table III), which indicates that one H_2O_2 molecule reacts with two coppers at the active site of *S. glaucescens* tyrosinase.

A) N62 (His 62 ⟶ Asn 62)

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                Phe Leu Pro Trp | His | Arg Arg Tyr      wild-type
                Phe Leu Pro Trp | Asn | Arg Arg Tyr      mutant

                       5'-C TGG | AAC | CGC AGA TA-3'    mutagenic primer (KL-9)
                                  *
          ....TCC CTG CCC TGG | CAC | CGC AGA TAC ...     tyrosinase gene
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B) N189 (His 189 ⟶ Asn 189)

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                Gly Val Asn Leu | His | Asn Arg Val      wild-type
                Gly Val Asn Leu | Asn | Asn Arg Val      mutant

                       5'-C CTG | AAC | AAC CGC GT-3'    mutagenic primer (KL-11)
                                  *
          ....GGC GTC AAC CTG | CAC | AAC CGC GTC ...     tyrosinase gene
```

FIGURE 3: Design of the primers KL-9 and KL-11 to introduce the mutations His-62 to Asn-62 (A) and His-189 to Asn-189 (B). The coding strand of the tyrosinase gene is shown (Huber et al., 1985). For the annealing reaction with the mutagenic primers, the noncoding strand was used as template. Boxes indicate the position of the substitution and asterisks the nucleotides changed.

Table IV: Comparison of the Physicochemical Properties of Wild Type and Mutants

	enzymatic act. (units/mg)	copper content (mol/mol of enzyme)	$g_\perp$	$g_\parallel$	$A_\parallel$ (cm^{-1})
wild type	2100	1.8	EPR silent		
mutant N62	0	0.7	2.23	2.048	0.0202
mutant N189	0	1.0	2.23	2.045	0.0202
metapo-N_3^- [a]			2.24	2.058	0.0152

[a] *B. canaliculatum* hemocyanin (Himmelwright et al., 1979).

The copper content of the enzyme was determined with a spectrophotometric assay (see Materials and Methods). This yielded a value of 1.8 mol of copper/mol of enzyme (Table IV). At 77 K most of the copper (95%) is present in an EPR-nondetectable form, indicating a strong antiferromagnetic coupling of the two copper ions.

Characterization of the Mutants His-62 → Asn (N62) and His-189 → Asn (N189). On the basis of the sequence comparison published earlier (Huber et al., 1985), histidine-62 and -189 in *S. glaucescens* tyrosinase are likely candidates as copper ligands. They were therefore changed to asparagine by application of site-directed mutagenesis. Figure 3 shows part of the amino acid sequences of both mutants together with the corresponding nucleotide sequences and the mutagenic primers. In both mutants, the histidine codon CAC was substituted by the triplet AAC coding for asparagine. On melanin indicator plates (Materials and Methods; Hintermann et al., 1985), both mutants N62 and N189 gave white colonies exclusively instead of black ones normally found with the wild-type gene.

The two mutant proteins were purified exactly as described for the wild-type enzyme (Figure 1). Some of their properties are shown in Table IV and compared to those of the native enzyme. As expected from the melanin indicator plates (See above), both mutants are devoid of any enzymatic activity. Despite this, the far-UV CD spectra of wild-type enzyme and of both mutants were indistinguishable, suggesting that the mutations do not introduce changes in tertiary structure (data not shown). The mutants contain only about 1 mol of copper/mol of enzyme. These results are further supported from the absorption spectra obtained after the addition of a 10-fold molar excess of hydrogen peroxide (Figure 4), showing neither an absorption at 345 nm nor one at 640 nm as typically found

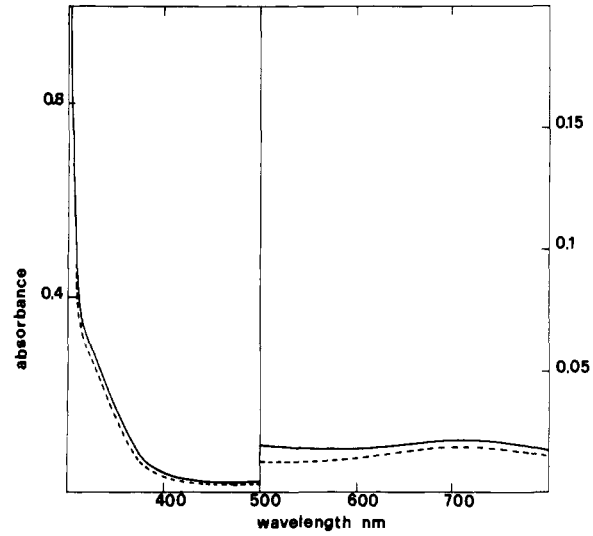

FIGURE 4: Absorption spectra of *S. glaucescens* tyrosinase mutant N62 (190 μM in 10 mM Tris-HCl, pH 8.6/0.1 M NaCl at 4 °C). The spectrum of freshly isolated enzyme (--) is compared to the one obtained after the addition of a 10-fold molar excess of H_2O_2 (—). For the mutant N189, essentially the same spectra were obtained.

for the wild-type enzyme (Figure 2). The only observable feature in the spectra obtained both from the freshly isolated protein and after the addition of H_2O_2 is a band at 710 nm which can be ascribed to Cu(II) d–d transitions. The extinction coefficient of $\epsilon_{710} = 115\ M^{-1}\ cm^{-1}$ suggests the presence of about one copper atom per protein molecule as calculated from a comparison with the value found for the met form from *N. crassa* tyrosinase (Lerch, 1981).

The EPR spectra of the mutants N62 and N189 are shown in Figure 5. They have a more or less axial symmetric appearance with the parameters given in Table IV. The values are very similar for both mutants, suggesting that the remaining copper is in a very similar ligand environment. The double integration of the EPR spectra revealed that in both mutants more than 95% of the copper present is EPR active.

Discussion

Tyrosinase from the bacteria *S. glaucescens* has been reported to contain a mononuclear copper site (Lerch & Ettlinger, 1972). In the light of recent findings (Lerch, 1981), the state of copper was reinvestigated. To this end, *S. glaucescens* tyrosinase was isolated from a high level expressing

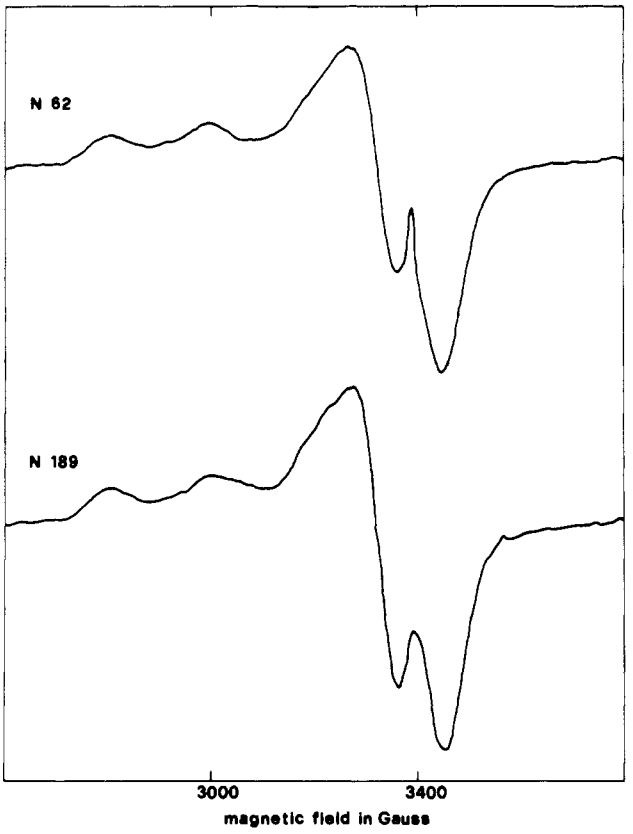

FIGURE 5: EPR spectra of the mutants N62 (copper concentration 0.32 mM) and N189 (1.3 mM) at 77 K and 9.49 GHz (10 mM Tris-HCl, pH 8.6/0.1 M NaCl). The microwave power was 1.59 mW, the frequency modulation was 100 kHz, and the field modulation was 45 G.

strain which allowed the purification of about 20 mg of enzyme from 5 g of mycelia. Both purity and yield were comparable to those of an earlier published procedure (Lerch & Ettlinger, 1972). The measurement of the copper content and the H_2O_2 titration experiment showed the presence of two coppers at the active site of this enzyme. They are strongly antiferromagnetically coupled as found previously for *N. crassa* and mushroom tyrosinases (Deinum et al., 1976; Schoot Uiterkamp & Mason, 1973). However, the spectrum of the oxy form from *S. glaucescens* tyrosinase is different from the one reported for the *N. crassa* enzyme (Lerch, 1976). The low-energy absorption at 600 nm is shifted to 640 nm in the bacterial enzyme. The difference is further borne out by a comparison of the CD spectra from *S. glaucescens* and *N. crassa* oxytyrosinases. The positive absorption at 520 nm in the fungal enzyme (Lerch, 1983) is shifted to 470 nm in the bacterial tyrosinase together with the appearance of an additional negative band at 575 nm. This suggests that the ligand environment of the copper ions in *S. glaucescens* tyrosinase is slightly different from the one in *N. crassa* tyrosinase. The CD properties measured for the oxy form of *S. glaucescens* tyrosinase are remarkably similar to those obtained for oxyhemocyanin from the mollusc *Busycon canaliculatum* (Himmelwright et al., 1980).

In order to identify the regions involved in copper binding in *S. glaucescens* tyrosinase, different amino acids where exchanged by site-directed mutagenesis. Those residues possibly necessary for copper binding were inferred from the comparison of the primary structures of tyrosinases and hemocyanins from different sources (Huber et al., 1985; Lerch et al., 1986). Therefore, His-62 and His-189 were exchanged to asparagine. This mutation should not significantly disturb the protein secondary structure since both amino acids occupy approximately the same space (Knowles, 1987). Furthermore, the amide nitrogen group is not thought to be a good ligand for either Cu(I) or Cu(II). Both mutants showed drastically changed physicochemical properties: (i) no enzymatic activity; (ii) no reaction with hydrogen peroxide; (iii) only one copper bound per molecule. The fact that most of this copper is EPR detectable confirms that one copper from either the Cu(A) site (N62) or the Cu(B) site (N189) has been selectively removed. The mutants can therefore be viewed as met-apo derivatives of tyrosinase. The measured EPR parameters compare very well with those from the met-apo derivatives of *B. canaliculatum* hemocyanin (Table IV; Himmelwright et al., 1979) and *A. bisporous* tyrosinase (Fry & Strothkamp, 1983). By use of the $g_{\parallel}$ and $A_{\parallel}$ values (Table IV) and the energies of the d–d transition, the copper in both mutants can be defined to be in a slightly distorted tetragonal ligand environment (Solomon, 1981). Applying the correlation between $A_{\parallel}/g_{\parallel}$, ligand type, and geometry in copper complexes (Peisach & Blumberg, 1974; Sakaguchi & Addison, 1979), possible copper ligands in *S. glaucescens* tyrosinase are either three nitrogen and one oxygen or two nitrogen and two oxygen atoms. The presence of three nitrogen donor atoms from histidyl residues as nearest neighbors around each of the copper ions was clearly established for *P. interruptus* deoxyhemocyanin (Gaykema et al., 1984).

Taken together, these data strongly suggest that His-62 and -189 are copper ligands in *S. glaucescens* tyrosinase. The outstanding sequence similarity of the region containing His-189 in *S. glaucescens* tyrosinase to that in hemocyanins and other tyrosinases argues that the corresponding histidyl residue in the other proteins is also a copper ligand. The mutant N62 defines a novel Cu(A) region different from those of arthropodan hemocyanins and *N. crassa* tyrosinase. This indicates that the Cu(B) site has been conserved during evolution whereas the Cu(A) site is variable.

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Registry No. Cu, 7440-50-8; L-His, 71-00-1; L-Asn, 70-47-3; tyrosinase, 9002-10-2.

REFERENCES

Aasa, R., & Vänngard, T. (1975) *J. Magn. Reson. 19*, 308–315.

Baumann, R., & Kocher, H. R. (1976) in *2nd International Symposium on Genetics of Industrial Microorganisms* (MacDonald, K. D., Ed.) pp 535–551, Academic, London.

Crameri, R., Hintermann, G., & Hütter, R. (1984) *Can. J. Microbiol. 30*, 1058–1067.

Deinum, J., Lerch, K., & Reinhammar, B. (1976) *FEBS Lett. 69*, 161–164.

Fling, M., Horowitz, N. H., & Heinemann, S. F. (1963) *J. Biol. Chem. 238*, 2045–2053.

Fry, D. C., & Strothkamp, G. (1983) *Biochemistry 22*, 4949–4953.

Gaykema, W. P. J., Hol, W. G. J., Vereijken, N. M., Soeter, M. N., Bak, H. J., & Beintema, J. J. (1984) *Nature (London) 309*, 23–29.

Himmelwright, R. S., Eickman, N. C., & Solomon, E. I. (1979) *J. Am. Chem. Soc. 101*, 1575–1586.

Himmelwright, R. S., Eickman, N. C., LuBien, C. D., & Solomon, E. I. (1980) *J. Am. Chem. Soc. 102*, 5378–5388.
Hintermann, G., Zatchej, M., & Hütter, R. (1985) *Mol. Gen. Genet. 200*, 422–432.
Huber, M., Hintermann, G., & Lerch, K. (1985) *Biochemistry 24*, 6038–6044.
Jolley, R. L., Evans, L. H., Makino, N., & Mason, H. S. (1974) *J. Biol. Chem. 249*, 335–345.
Joyce, C. M., & Grindley, N. D. F. (1984) *J. Bacteriol. 158*, 636–644.
Kieser, T., Hopwood, D. A., Wright, H. M., & Thompson, C. J. (1982) *Mol. Gen. Genet. 185*, 223–238.
Knowles, J. R. (1987) *Science (Washington, D.C.) 236*, 1252–1258.
Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488–492.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lerch, K. (1976) *FEBS Lett. 69*, 157–160.
Lerch, K. (1981) *Met. Ions Biol. Syst. 13*, 143–186.
Lerch, K. (1983) *Mol. Cell. Biochem. 52*, 125–138.
Lerch, K., & Ettlinger, L. (1972) *Eur. J. Biochem. 31*, 427–437.
Lerch, K., Huber, M., Schneider, H.-J., Drexel, R., & Linzen, B. (1986) *J. Inorg. Biochem. 26*, 213–217.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Mandel, M., & Higa, A. (1970) *J. Mol. Biol. 53*, 159–162.
Mason, H. S. (1965) *Annu. Rev. Biochem. 34*, 594–634.
Miller, J. H. (1972) in *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Miyoshi, K., Huang, T., & Itakura, K. (1980a) *Nucleic Acids Res. 8*, 5491–5505.
Miyoshi, K., Miyaka, T., Hozumi, T., & Itakura, K. (1980b) *Nucleic Acids Res. 8*, 5473–5490.
Moore, S., & Stein, W. H. (1963) *Methods Enzymol. 6*, 819–831.
Peisach, J., & Blumberg, W. E. (1974) *Arch. Biochem. Biophys. 165*, 691–708.
Sakaguchi, U., & Addison, A. W. (1979) *J. Chem. Soc., Dalton Trans.*, 600–608.
Sanger, F., Nicklen, S., & Coulsen, A. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5468.
Schoot Uiterkamp, A. J. M., & Mason, H. S. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 993–996.
Shibahara, S., Tomita, Y., Sakakura, T., Nager, C., Chaudhuri, B., & Müller, R. (1986) *Nucleic Acids Res. 14*, 2413–2427.
Skotland, T., & Ljones, T. (1979) *Eur. J. Biochem. 94*, 145–151.
Solomon, E. I. (1981) in *Copper Proteins* (Spiro, T. G., Ed.) pp 42–108, Wiley-Interscience, New York.
Thompson, C. J., Ward, J. M., & Hopwood, D. A. (1980) *Nature (London) 286*, 525–527.
Thompson, C. J., Ward, J. M., & Hopwood, D. A. (1982) *J. Bacteriol. 151*, 668–677.
Yanisch-Perron, C., Vieira, J., & Messing, J. (1985) *Gene 33*, 103–119.
Zoller, M. J., & Smith, M. (1983) *Methods Enzymol. 100*, 468–500.

Disulfide-Linked Dimer of Oncomodulin: Comparison to Calmodulin†

Bulent Mutus,*,‡ Elizabeth J. Palmer,‡ and J. P. MacManus§
Department of Chemistry and Biochemistry, University of Windsor, Windsor, Ontario N9B 3P4, Canada, and Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario K1A 0R6, Canada
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ABSTRACT: Oncomodulin, an oncofetal Ca^{2+}-binding protein, contains a single Cys residue in position 18 of its primary structure. The reactivity of the Cys-18 thiol has been probed with 5,5′-dithiobis(2-nitrobenzoate) (NbS_2). The kinetics of the reaction indicate that the thiol group is ~10-fold more reactive in the presence of Ca^{2+} than in its absence. Evidence presented here shows that oncomodulin can dimerize by intermolecular disulfide formation via the Cys-18 thiol. The kinetics of dimer formation indicate that the second-order rate constant for this reaction is ~6-fold higher than that observed for the reaction of the Cys-18 thiol with NbS_2, possibly indicating that intermolecular electrostatic interactions precede disulfide formation. The disulfide-linked dimer of oncomodulin appears to be more similar to calmodulin than oncomodulin since the dimer displayed "calmodulin-like" affinity for the amphiphilic peptide melittin. In addition, oncomodulin dimer was shown to activate two calmodulin-dependent enzymes, cyclic nucleotide phosphodiesterase and calcineurin phosphatase, with the activity constants of 63 and 1 nM, respectively, indicating that these enzymes have different domain contact requirements for activation.

Oncomodulin is a Ca^{2+}-binding protein (M_r 11 700) originally isolated from rat Morris hepatomas (MacManus, 1979). It has since been detected in tumors from mice, guinea pigs, and humans (MacManus et al., 1983, 1984). The detection of this protein in the rat placenta indicates that oncomodulin is oncodevelopmental in origin; that is, it is expressed in early development and upon transformation (Brewer & MacManus, 1985; MacManus et al., 1985).

The primary structure of oncomodulin (ONC)[1] shows extensive homology to the β-parvalbumin subclass of the troponin

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‡ University of Windsor
§ National Research Council of Canada.

[1] Abbreviations: NbS_2, 5,5′-dithiobis(2-nitrobenzoate); ONC, oncomodulin; ONC-d, disulfide-linked oncomodulin dimer; CaM, calmodulin; PDE, cyclic nucleotide phosphodiesterase; CaN, calcineurin; PV, parvalbumin; TnC, troponin C; DTT, dithiothreitol; PNPP, *p*-nitrophenyl phosphate; PAGE, polyacrylamide gel electrophoresis, HPSEC, high-performance size-exclusion chromatography.

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