Stabilization of the oxy form of tyrosinase by a single conservative amino acid substitution

Martin P. JACKMAN,* Marcel HUBER, Alex HAJNAL and Konrad LERCH†

Biochemisches Institut der Universität Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

Asp-208 of *Streptomyces glaucescens* tyrosinase (an invariant residue in the Cu_B-binding region of tyrosinases and haemocyanins) was conservatively substituted by glutamic acid. Although having little effect on spectroscopic or kinetic properties of the enzyme, the mutation greatly decreased the lability of Cu-bound O₂. A rationalization for these results is given, based on the crystal structure of *Panuliris interruptus* haemocyanin in the conserved Cu_B-binding region.

INTRODUCTION

The mono-oxygenase tyrosinase (EC 1.14.18.1) (Ty) is responsible for the formation of melanin and related pigments by catalysing the *o*-hydroxylation of monophenols and the two-electron oxidation of *o*-diphenols to *o*-quinones [1,2]. The enzyme is ubiquitously distributed, from Gram-positive bacteria to man [3].

Physicochemical studies have shown that the enzyme's active site contains a coupled binuclear copper complex possessing remarkably similar spectral properties to those of haemocyanins (Hc), the O₂-transport protein of molluscs and arthropods [4–7]. Thus the naturally occurring oxy (Cu^{II}₂O₂²⁻), met (Cu^{II}₂) and deoxy (Cu^I₂) forms as well as the carbonyl and azide derivatives of both copper proteins have been described [3,8].

Three-dimensional structural information is at present restricted to Hc from the arthropod *Panulirus interruptus*. The refined structure of deoxy-Hc [9,10] reveals a pseudo-two-fold active-site symmetry with each of the copper atoms, designated Cu_A and Cu_B, ligated by three histidine residues. Comparison of many Ty and Hc primary structures [10–12] has led to the proposal of a common Cu_B-binding region for these proteins, encompassing the three putative histidine ligands and a highly conserved sequence of 19 residues that constitutes α -helix 2.6 (residues 377–395) in the *P. interruptus* Hc crystal structure [10]. In contrast, the Cu_A-binding sites of Ty and Hc appear to be rather variable [11]. These observations have been reinforced by site-directed mutagenesis experiments on *Streptomyces glaucescens* Ty [13,14]. Replacement of the histidine residues corresponding to the Cu_B ligands of *P. interruptus* Hc by asparagine or glutamine resulted in loss of active-site copper and drastically decreased activity in the mutant enzymes, implying their involvement as Cu ligands. Mutation of further targeted histidine residues has allowed the tentative assignment of a novel Cu_A-binding site in this enzyme [13,14].

Here we report the conservative substitution of Asp-208 of *S. glaucescens* Ty by glutamic acid (mutant D208E). This invariant aspartic acid residue lies within the highly conserved region, preceding Cu_B-ligand His-215 by seven residues. The spectroscopic and kinetic properties of native and mutant enzymes are compared, and the results cautiously interpreted with reference to the crystal structure of *P. interruptus* Hc.

MATERIALS AND METHODS

Mutagenesis and expression of the tyrosine gene

Site-directed mutagenesis was performed by the Kunkel method [15] with the use of the uracil-containing 480 bp *Pst*I–*Bam*HI fragment of the *S. glaucescens* Ty gene [16] cloned into M13mp19, and the anti-sense mutagenic primer 5'-CACCGGCTCGTTGGG-3'. Details of mutagenesis and amplification have been published previously [13,14]. The identity of the mutation was confirmed by chain-termination DNA sequencing [17].

Tyrosinase expression and purification

The 2.2 kbp *Kpn*I fragment containing the complete Ty gene was inserted into *Streptomyces* vector pMEA4 [18] and expressed in *S. glaucescens* by following literature procedures [13]. Mutant and native enzymes were purified by using a published method [14] in which CuCl₂ (10 μ M) was present throughout chromatography in order to suppress loss of copper from the active site. After the purification, extraneous Cu(II) was removed by passing the protein sample through a Sephadex G-25 column equilibrated in copper-free 10 mM-Tris/HCl buffer, pH 8.6. The resulting enzymes were purified to homogeneity, judged from SDS/12.5%-PAGE gels [19] stained with Coomassie Blue G-250. Samples were used immediately for further experiments, since storage results in slow pigmentation and inactivation. Protein concentrations were calculated by using the absorption coefficient ϵ_{280} 8.21×10^4 M⁻¹·cm⁻¹ [14] determined for native Ty.

Spectroscopic and kinetic measurements

All spectroscopic measurements were made in 10 mM-Tris/HCl buffer, pH 8.6. Copper concentrations were determined with the use of the Cu(I)-chelator bathocuproinedisulphonic acid [20]. Measurements were confined to the range 0.4–10 μ g of Cu/ml, within the linear region of a calibration curve constructed with standard dilutions of CuCl₂ (Titrisol; Merck). Maximal oxy-enzyme formation was achieved by adding hydroxylamine in 3-fold molar excess over copper. Absorption spectra were measured at 10 °C on a Kontron Uvicon 860 spectrometer. C.d. spectra of the oxy-enzymes and luminescence spectra of the carbonyl derivatives were obtained as previously described [13,14]. In the

Abbreviations used: Ty, tyrosinase; Hc, haemocyanin.

* To whom correspondence should be sent, at present address: Friedrich Miescher Institut, P.O. Box 2543, CH-4002 Basel, Switzerland.

† Present address: Givaudan Forschungsgesellschaft, Überlandstrasse 138, CH-8600 Dübendorf, Switzerland.

Scheme 1. Equilibrium between met, oxy and deoxy forms of Ty

Fig. 2. Comparison of oxy-enzyme labilities at 10 °C

●, Native Ty (9.1 μM); ○, mutant D208E (9.8 μM). The time point of catalase addition is indicated by an arrow.

under the conditions described in the Materials and methods section, with apparent rate constants of $4.4 \times 10^{-4} \text{ min}^{-1}$ in the absence and $1.07 \times 10^{-3} \text{ min}^{-1}$ in the presence of 40 nM-catalase. The faster decay in the presence of catalase demonstrates that an equilibrium between free and bound peroxide had been established. As expected, catalase increased the rate of decay of oxy-enzyme by removing O_2^{2-} , thus shifting the equilibrium in Scheme 1 in favour of met-Ty. For mutant D208E, however, the A_{345} was constant over the whole time course of the experiment, both before and after the addition of catalase. Setting an upper limit of $1 \times 10^{-5} \text{ min}^{-1}$ for the apparent decay rate constant, this represents at least a 40-fold decrease in lability of enzyme-bound peroxide.

In contrast with the Cu-bridged nature of bound peroxide, Ty and Hc each bind a single CO molecule in a terminal non-bridging manner [31,32], giving rise to an unusual fluorescence

Fig. 3. Active-site structure of *P. interruptus* Hc

The Figure shows a view of the α -helices bearing the Cu ligands. The hydrogen-bonding interactions between Arg-271 and Asp-377 of α -helix 2.6 are indicated.

spectrum when excited at 275 nm. It has been proposed that CO binding occurs at Cu_A [9,14,33]. Interestingly, the carbonyl luminescence spectrum of D208E is almost identical with that of native Ty (Table 1), suggesting that the Cu_A environment is not perturbed by the effect of mutation.

Kinetic properties

Kinetic rate constants for reaction of native and mutant Ty with monophenol and diphenol substrates and O_2 are presented in Table 2. The mutation clearly did not affect the K_m of organic substrates and caused only a nominal decrease in catalytic rate constant of 2–3-fold for both *o*-hydroxylation and two-electron oxidation. A similar effect was observed for the reaction with O_2 with L-3,4-dihydroxyphenylalanine as organic substrate. It should be noted that the equilibrium described in this case is for binding of molecular O_2 and not of peroxide (see Scheme 1), perhaps explaining why a larger effect is not observed.

In summary, the conservative replacement D208E greatly enhances the stability of the oxy-enzyme with respect to the met form and free peroxide, while causing only small perturbations in the spectroscopic and kinetic properties of the enzyme. A tentative explanation for these results can be found by examining the crystal structure of *P. interruptus* Hc [9,10]. α -Helix 2.6, containing Cu ligand His-384, appears to be 'tethered' at its base

Table 2. Kinetic parameters for native tyrosinase and mutant D208E with a range of substrates

Conditions are as described in the Materials and methods section. Abbreviations: L-Dopa, L-3,4-dihydroxyphenylalanine; 3,4-Dhpa, 3,4-dihydroxyphenylpropionic acid; 4-Hpa, 4-hydroxyphenylpropionic acid.

Substrate	Native enzyme			Mutant D208E		
	$k_{\text{cat.}}$ (s^{-1})	K_m (mM)	$10^5 \times k_{\text{cat.}}/K_m$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)	$k_{\text{cat.}}$ (s^{-1})	K_m (mM)	$10^5 \times k_{\text{cat.}}/K_m$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)
L-Dopa	1260	5.8	2.2	680	6.3	1.1
3,4-Dhpa	1470	1.3	12	930	1.3	7.2
L-Tyrosine	82	2.4	0.35	22	2.0	0.11
4-Hpa	193	0.4	4.8	100	0.5	2.0
O_2	660	0.084	79	340	0.071	48

by the salt bridge formed between Asp-377 and Arg-271 (Fig. 3). The sequence forming α -helix 2.6 is highly conserved between Hc and Ty (Fig. 1), suggesting that a similar tethering mechanism may operate in *S. glaucescens* Ty. Extending the length of the tether by inserting a $-\text{CH}_2-$ group (mutant D208E) could cause strain to be transmitted through the rigid α -helix to Cu ligand His-215, altering the Cu_B geometry without affecting the Cu_A environment. Such an effect might be expected to influence the binding of bridged ligands (e.g. peroxide) more strongly than that of non-bridged ligands such as CO, consistent with the spectroscopic results observed here. However, this proposal should be treated with caution: the region of *P. interruptus* Hc sequence containing Arg-271 is not conserved in Ty [11], and thus a putative hydrogen-bonding partner for Asp-208 of Ty cannot readily be identified. A conclusive interpretation must await structural data for a closely related Ty molecule.

We gratefully acknowledge the Royal Society of Great Britain and the Roche Research Foundation for financial support (M. P. J.), and Dr. W. Hol for providing the crystal-structure co-ordinates of *P. interruptus* Hc.

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Received 6 August 1991/1 November 1991; accepted 13 November 1991