Spectroscopic studies of the reaction between bovine serum amine oxidase (copper-containing) and some hydrazides and hydrazines

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The carbonyl cofactor of bovine serum amine oxidase, recently identified as pyrroloquinoline quinone [Ameyama, Hayashi, Matsushita, Shinagawa & Adachi (1984) *Agric. Biol. Chem.* **48**, 561–565; Lobenstein-Verbeek, Jongejan, Frank & Duine (1984) *FEBS Lett.* **170**, 305–309], reacts stoichiometrically and irreversibly with hydrazides of phenylacetic acid and of benzoic acid. With the phenylacetic hydrazides a reversible intermediate step was detected by competition with substrate, carbonylic reagents or phenylhydrazine, a typical inhibitor of the enzyme. All hydrazides form an intense broad band with maximum absorbance in a narrow wavelength range (350–360 nm), irrespective of the acyl group, suggesting that the transition is located on the organic cofactor. A different situation is found with some phenylhydrazines, where extended conjugation can occur between the cofactor and the phenyl π -electron system via the azo group, as shown by the lower energy and higher intensity of the transition. In this case the transition is sensitive to substituents in the phenyl ring. The c.d. spectrum of the adducts is influenced by the type of hydrazide (derived from phenylacetic acid or benzoic acid), by pH and by *NN*-diethyldithiocarbamate binding to copper, probably as a result of shifts of equilibria between hydrazone–azo tautomers.

INTRODUCTION

Bovine serum amine oxidase (copper-containing) (BSAO) (EC 1.4.3.6) catalyses the oxidative deamination of primary amines to the corresponding aldehydes with production of ammonia and hydrogen peroxide. BSAO is a dimer composed of two subunits, of probably identical primary structure as suggested by electrophoresis under denaturing conditions and by tryptic peptide mapping (Achee *et al.*, 1968). Two e.p.r.-detectable Cu(II) ions and one titrable organic group, covalently bound to the enzyme and responsible for a broad absorption band at 480 nm, are present in the active sites. The chemical nature of the chromophore has been a controversial matter for a long time. Optical modifications, induced by treatment with hydrazine and other carbonyl reagents, indicated that it might be pyridoxal 5'-phosphate (PLP) (Finazzi-Agrò *et al.*, 1977), but unequivocal evidence was never produced. Recently two independent groups have concluded that the chromophore is pyrroloquinoline quinone (PQQ), on the basis of the similarity of chromatographic and spectral properties between the 2,4-dinitrophenylhydrazine adducts of synthetic PQQ and of the cofactor isolated from the enzyme (Ameyama *et al.*, 1984; Lobenstein-Verbeek *et al.*, 1984).

Only one molecule of the prosthetic group is present per dimeric enzyme. The strongest evidence is the reaction with phenylhydrazine, which irreversibly binds and fully inhibits at the ratio of one phenylhydrazine molecule per dimeric enzyme. The binding causes bleaching of the absorption at 480 nm and the appearance of a new

intense absorption band at 445 nm (Rinaldi *et al.*, 1983).

Recently some hydrazides of pyrrolybenzoic acid and pyrrolyphenylacetic acid have been synthesized in view of a possible pharmacological use as antihypertensive and antifibrotic agents, and their action on both FAD and copper-containing amine oxidase has been tested (Artico *et al.*, 1988). They were found to act as highly specific inhibitors of BSAO, very probably by binding to the carbonyl cofactor. Since no detailed studies exist on the reaction between copper-containing amine oxidases and hydrazides, we decided to investigate in more detail the reaction between BSAO and these hydrazides to obtain additional information on the binding site and its reactions. The study was extended to isoniazid, the hydrazide of isonicotinic acid widely used in the treatment of tuberculosis (Carrington *et al.*, 1984), and to simple derivatives of benzoic acid and phenylacetic acid to test the effect on reactivity and spectroscopic properties of different hydrazides, respectively in view of possible pharmacological use and of an understanding of the protein molecular structure.

MATERIALS AND METHODS

BSAO was purified according to the method of Turini *et al.* (1982) with minor modifications. The $(\text{NH}_4)_2\text{SO}_4$ fraction was loaded on to a column of CM-cellulose equilibrated with 10 mM-potassium phosphate buffer, pH 5.8. Haemoglobin was retained and the enzyme was eluted. The protein concentration was deter-

Abbreviations used: BSAO, bovine serum amine oxidase (copper-containing); PLP, pyridoxal 5'-phosphate; PQQ, pyrroloquinoline quinone.

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Table 1. Reactivity of hydrazides with BSAO and spectroscopic properties of the adducts

Abbreviations: P, phenylacetic acid hydrazide; B, benzhydrazide.

Compound	U.v.-visible		C.d.		Hydrazide/ phenylhydrazine competition	Reaction time
	ϵ ($M^{-1} \cdot cm^{-1}$)	λ_{max} (nm)	$\Delta\epsilon$ ($M^{-1} \cdot cm^{-1}$)	λ_{max} (nm)		
4-(1-Pyrryl)P	16 700	359	-21.2	395	55 %	< 1 min
Acetone derivative	17 000	359	-20.5	395	38 %	$\frac{1}{2}$ h
3-(1-Pyrryl)P	15 600	375 $\rightarrow$ 364	-27.9	395	30 %	< 1 min
4-(1-Pyrryl)B	23 700	362	+17.4	363	5 %	1 h
3-(1-Pyrryl)B	33 100	354	+33.3	360	None	12 h
B	28 000	359	-14.9	400	-	2 h
P	12 000	363	-21.8	395	-	1 h
4-Cl-P	17 000	359	-9.4	404	-	2 h
			+6.1	350		
4-Cl-P (pH 5.8)	15 000	359	-4.4	413		
			+9.1	360		
4-NH ₂ -P	14 800	360	-9.5	404	-	2 h
			+4.5	360		
4-(CH ₃) ₂ N-P	9800	359	-9.8	404	-	2 h
			+8.8	360		
Isoniazid	22 500	347	+27.6	352	-	4 h
Semicarbazide	22 700	360	+28.1	365	-	1 h

mined from the absorbance at 280 nm by using $a = 1.74 \text{ litre} \cdot g^{-1} \cdot cm^{-1}$ (Suzuki *et al.*, 1983) and by the biuret method in accordance with Goa (1953). The concentration of the carbonyl cofactor was measured at 445 nm by reaction with phenylhydrazine ($\Delta\epsilon = 38000 \text{ M}^{-1} \cdot cm^{-1}$) (Suzuki *et al.*, 1983). Enzyme activity was followed at 37 °C by monitoring spectrophotometrically at 250 nm the oxidation of benzylamine (3 mM) to benzaldehyde ($\epsilon = 12500 \text{ M}^{-1} \cdot cm^{-1}$). All experiments were carried out in 0.1 M-potassium phosphate buffer, pH 7.2, unless otherwise stated.

Phenylacetic hydrazide, 4-chlorophenylacetic hydrazide, 4-aminophenylacetic hydrazide and 4-dimethylaminophenylacetic hydrazide were synthesized by standard procedures. Isoniazid, benzylamine and PLP were purchased from Sigma Chemical Co., phenylhydrazine hydrochloride, semicarbazide hydrochloride, 2,4-dinitrophenylhydrazine and hydrazine sulphate were from Merck, and benzhydrazide was from Fluka. They were used without further purification. 3- and 4-(1-Pyrryl)phenylacetic hydrazide, acetone 4-(1-pyrryl)-phenylacetic hydrazone and 3- and 4-(1-pyrryl)benzhydrazide were synthesized in accordance with Artico *et al.* (1988).

Optical spectra were recorded on a Beckman DU-7 spectrophotometer. C.d. spectra were recorded on a Jasco 500 spectropolarimeter. E.p.r. spectra were recorded on a Varian E9 spectrometer.

RESULTS

Absorption spectra of BSAO-hydrazide derivatives

The stoichiometric addition of pyrrylbenzoic hydrazide and pyrrylphenylacetic hydrazide to BSAO induced spectral changes in the visible and near-u.v. region. An intense new absorption band was formed at about 360 nm (Table 1), where the hydrazides do not absorb appreciably. In Fig. 1 the spectra of the native enzyme

(spectrum *A*) and of its adduct with 4-(1-pyrryl)-phenylacetic hydrazide (spectrum *C*) are displayed together with that of the phenylhydrazine derivative for comparison (spectrum *B*). The appearance of the 360 nm band paralleled the decrease of the 480 nm absorption. Further addition of hydrazides up to 5-fold excess did not cause absorbance change. It was not possible to test higher concentrations owing to the low solubility of the pyrryl derivatives. The band around 480 nm, due to the organic cofactor (Suzuki *et al.*, 1983), was decreased to about the same extent by phenylhydrazine and 4-(1-pyrryl)phenylacetic hydrazide, but with benzhydrazides the decrease was less pronounced. No decrease at all was found with semicarbazide.

The reaction time was different for the hydrazides of phenylacetic acid and of benzoic acid. In the first case [3- and 4-(1-pyrryl)phenylacetic hydrazide] the formation of the adduct with BSAO was immediate, whereas hours were required in the latter case. The times required at 25 °C for complete reaction, i.e. attainment of maximum absorbance change after the addition of 1 equivalent of hydrazide, are reported in Table 1. Complete loss of enzymic activity was not always observed in these conditions, probably owing to some reversibility of the reaction caused by dilution. This point is examined in detail below under 'Reversibility'. With 3-(1-pyrryl)-phenylacetic hydrazide two spectroscopically different species were formed, one immediately after the addition with absorption band centred at 375 nm, the other after 2-3 min with maximum absorbance shifted to 364 nm. No spectral shift was observed with any of the other hydrazides. Substitution of the pyrryl moiety by hydrogen, chlorine, amino group or dimethylamino group had no effect on the stoichiometry or on the spectroscopic properties of the derivatives, but the reaction was slower. The substitution of the phenyl by a pyridyl group, as in isoniazid, or even the absence of the aryl moiety, as in semicarbazide, did not produce significant optical or

Fig. 1. Optical spectra of BSAO derivatives

Native BSAO (50 μ M) in 0.1 M-phosphate buffer, pH 7.2 (spectrum A), was treated with stoichiometric phenylhydrazine (spectrum B) or 4-(1-pyrryl)phenylacetic hydrazide (spectrum C).

kinetic difference in the adducts (Table 1). The acetone 4-(1-pyrryl)phenylacetic hydrazone also reacted with BSAO. The optical spectrum of the adduct was similar to that of the parent hydrazide, but the reaction was slower. These results indicate that in the presence of BSAO the acetone dissociated, allowing the enzyme to react with the hydrazide.

Table 2 reports the results obtained with a series of hydrazines. The reaction occurred within mixing time at stoichiometric concentrations, except with 2,4-dinitrophenylhydrazine. In the latter case the reaction was much slower and could be accelerated by incubation at 30 °C with excess hydrazine. The unused reagent was then removed by dialysis.

C.d. spectra of BSAO-hydrazide derivatives

Position, intensity and sign of the c.d. bands, measured at the end point of reaction, are also reported in Tables

1 and 2. Phenylacetic hydrazides produced a negative Cotton effect at 395 nm with BSAO, whereas benzhydrazides, isoniazid and semicarbazide caused a positive Cotton effect at about 360 nm. Some hydrazides of phenylacetic acid produced two Cotton effects: a negative one at about 400 nm and a positive one at 360 nm, the peak-to-peak intensity being comparable with those of the others. Unsubstituted benzhydrazide showed a negative Cotton effect at 400 nm similar to that of the phenylacetic hydrazides.

The c.d. spectra were also measured at pH 5.8. With some derivatives reversible changes were observed, as shown in Table 1 for 4-chlorophenylacetic hydrazide. In the corresponding optical spectrum a reversible decrease of intensity of the 360 nm absorption band was observed, in spite of the increased intensity of the c.d. effect at this wavelength. No change was seen with the benzhydrazide derivatives, with a positive c.d. band at 360 nm.

E.p.r. experiments: interaction of hydrazides with the copper sites

The binding of hydrazides affected the copper sites, as shown by the e.p.r. spectra of native and treated enzyme performed at 77 K and 9 GHz frequency (Fig. 3, spectra A and B). The presence of hydrazides bound to the organic cofactor did not prevent the copper from reacting with metal-binding inhibitors such as *NN*-diethyldithiocarbamate. In particular, the typical e.p.r. spectrum produced by reaction with *NN*-diethyldithiocarbamate was modified by the presence of the hydrazide. Fig. 2 shows the separate effects of 3-(1-pyrryl)phenylacetic hydrazide and *NN*-diethyldithiocarbamate (spectra B and D) respectively and the combined effect of the two reagents (spectrum C). The corresponding c.d. spectra are displayed in Fig. 3, showing a decrease of the hydrazide negative peak at 395 nm and formation of a new negative band at 330 nm, due to the hydrazide, whereas *NN*-diethyldithiocarbamate forms a positive peak at 370 nm with the native protein. In the visible-region spectrum the 364 nm band was shifted to about 350 nm in the *NN*-diethyldithiocarbamate-bound derivative, without significant change of intensity, besides the copper-*NN*-diethyldithiocarbamate charge-transfer-band contribution (Morpurgo *et al.*, 1987).

Table 2. Reactivity of hydrazines with BSAO and spectroscopic properties of the adducts

Abbreviations: Hy, hydrazine; PHy, phenylhydrazine; BHy, benzylhydrazine.

Compound	U.v.-visible		C.d.		Reaction time
	ϵ ($M^{-1} \cdot cm^{-1}$)	λ_{max} (nm)	$\Delta\epsilon$ ($M^{-1} \cdot cm^{-1}$)	λ_{max} (nm)	
PHy	38 000	446	+12.1	448	< 1 min
2,4-Dinitro-PHy	44 000	470	+16.1	470	*
4-Cl-PHy	42 000	450	+16.4	448	< 1 min
Hy	20 200	330	-15.2	345	< 1 min
	8400	410	+8.0	455	
BHy†	22 000	405	+34.5	408	< 1 min
9-Acridino-Hy‡	86 600	495			
	98 000	525			

* Incubated for 24 h at 30 °C.

† The spectra were recorded immediately after BHy addition, since fading occurred with time.

‡ Data taken from Lamkin *et al.* (1988).

Fig. 2. E.p.r. spectra of 3-(1-pyrryl)phenylacetic hydrazide- and *NN*-diethyldithiocarbamate-treated BSAO

Native BSAO ($38\ \mu\text{M}$) in 0.1 M-phosphate buffer, pH 7.2 (spectrum A), was treated with $75\ \mu\text{M}$ -3-(1-pyrryl)-phenylacetic hydrazide (spectrum B), with $75\ \mu\text{M}$ 3-(1-pyrryl)phenylacetic hydrazide and 10 mM-*NN*-diethyldithiocarbamate (spectrum C) and with *NN*-diethyldithiocarbamate (spectrum D). Samples B–D were incubated for 36 h at 5°C with reagents and then dialysed against buffer.

Fig. 3. C.d. spectra of 3-(1-pyrryl)phenylacetic hydrazide- and *NN*-diethyldithiocarbamate-treated BSAO

The samples were the same as in Fig. 3.

Reversibility

The reaction with some hydrazides was in part reversed by dialysis against phosphate buffer or against a competing carbonyl group, such as 3 mM-acetaldehyde, which caused recovery of activity, decrease of the 360 nm band and re-appearance of the 480 nm band. Also, the substrate (benzylamine) acted as a competing group, as indicated by the increase of enzymic activity with time (Fig. 4) on incubation with substrate of the inhibited enzyme. When BSAO adducts with the fast-reacting hydrazides were dialysed against acetaldehyde immediately after full development of the 360 nm band, the reaction was almost completely reversed, whereas upon

Fig. 4. Benzylamine oxidase activity of BSAO

Curve A, 50 mM-benzylamine in 0.1 M-phosphate buffer, pH 7.2, and native $0.1\ \mu\text{M}$ -BSAO; curve B, incubated for 24 h with stoichiometric 4-(1-pyrryl)phenylacetic hydrazide; curve C, after 24 h dialysis against acetaldehyde. The temperature was 37°C .

Fig. 5. Optical spectra of PLP derivatives

Spectrum A, $50\ \mu\text{M}$ -PLP in 50 mM- H_2SO_4 ; spectrum B, $50\ \mu\text{M}$ -4-(1-pyrryl)phenylacetic hydrazide in 50 mM- H_2SO_4 ; spectrum C, the stoichiometric mixture after 3 h incubation.

dialysis against phosphate buffer, without addition of a competing carbonyl compound, only about 50% of the activity was recovered. The same samples lost with time the capacity to dissociate the hydrazide, although no change was detectable in their optical spectra. No reversibility was observed with the slowly reacting hydrazides at any stage of the reaction. These experiments confirm previous studies based only on recovery of enzymic activity (Artico *et al.*, 1988).

Strictly related to reversibility was the capability of the adducts to react with phenylhydrazine. By treatment with 1 mol of phenylhydrazine/mol of enzyme an absorption band at 445 nm was slowly formed, with lower intensity with respect to the native enzyme. At the same time the intensity of the hydrazide absorption band decreased to some extent. The decrease of the hydrazide absorption was more evident in the c.d. spectra owing to

the better resolution of the c.d. bands. The intensity of the phenylhydrazine optical band relative to that formed by the native protein (Table 1) roughly correlated with the degree of reversibility of the compounds upon dialysis. No reaction with phenylhydrazine occurred with the slowly reacting irreversible hydrazido derivatives.

Reaction of PLP and PQQ with hydrazides

PLP was reported to form an intense absorption band at 410 nm as a result of reaction with phenylhydrazine in acid solutions (Finazzi-Agrò *et al.*, 1977). In analogous conditions (50 mM-H₂SO₄) a band around 350 nm, with intensity in the 8000–10000 M⁻¹·cm⁻¹ range, was obtained with all hydrazides. A 3–4 h period was required to develop the band fully (Fig. 5). A different spectrum was obtained after 3–4 days' incubation of PLP and hydrazides in neutral solution, but this was immediately converted by acidification into the form obtained in acid medium. No reactivity could be observed with PQQ under the same conditions.

DISCUSSION

Previous kinetic studies showed that hydrazides of pyrrolylbenzoic acid and of pyrrolylphenylacetic acid act as inactivators of BSAO. The inactivation process was time-dependent, and, at early stages of the reaction, was in part reversed by dialysis against buffer. The presence in the dialysis medium of a carbonyl compound, e.g. acetaldehyde, improved the re-activation of the enzyme (Artico *et al.*, 1988). These findings are substantiated by the present investigation. BSAO was found to react with all hydrazides in 1:1 ratio. The time required for complete reaction varied and was generally faster with the pyrrolyl derivatives of phenylacetic acid than with those of benzoic acid or the derivatives of phenylacetic acid lacking the pyrrolyl group. Therefore the presence of a hydrophobic residue substituted in the phenyl ring and a longer aliphatic chain, or perhaps the greater flexibility provided by the presence of an additional –CH₂– group between the phenyl group and the hydrazido group, increased the reaction rate. With the fast-reacting hydrazides a reversible derivative was initially formed and was then converted into a species that did not dissociate from the active site, with consequent enzyme inactivation. As previously suggested (Artico *et al.*, 1988), the reaction appears to occur in at least two steps, only the first one reversible, the difference between 'reversible' and 'irreversible' inhibitors being the relative values of the kinetic constants:

The degree of reversibility was also measured by competition with phenylhydrazine, a fast-reacting inactivator of copper-containing amine oxidases for which no intermediate reversible steps can be isolated (Pettersson, 1985). The c.d. spectra (not shown) unequivocally demonstrate that phenylhydrazine is able to displace in part the 'reversible' hydrazides from BSAO. In contrast, no phenylhydrazine reaction was observed when BSAO had reacted with the 'irreversible' ones. The data show that in all instances a single inhibitor molecule was bound to the protein, very probably to the same carbonyl group.

The optical absorption band of the hydrazido adducts is rather broad, with maximum absorbance in a narrow spectral region around 360 nm, almost insensitive to the nature of R in the R–CO–NH–NH₂ and R–CH₂–CO–NH–NH₂ derivatives (Table 1). This finding is at variance with the wide variability of the absorption band in the derivatives of hydrazines (Table 2), occurring in general at longer wavelength and of higher intensity than those of the hydrazido derivatives. A more complicated pattern is shown by the c.d. spectra (Table 1), which with most benzhydrazides gave rise to a positive Cotton effect at about 360 nm, coincident with the optical absorption, and to a negative one at about 400 nm with phenylacetic hydrazides. Interestingly the phenylacetic hydrazides lacking the pyrrolyl group gave rise to both Cotton effects, with comparable total intensity. In all instances a single optical absorption band at 360 nm was detectable, but with an asymmetrical tail in the visible region (Fig. 1), suggesting the presence of more components. The c.d. spectra may be affected by the pH, as shown in Table 1 for the 4-chlorophenylacetic hydrazide. The increase, at low pH, of the positive effect at 360 nm at the expense of the negative one at 400 nm suggests the presence of a pH-dependent equilibrium between tautomeric forms absorbing at the two wavelengths. The study of this equilibrium is complicated by irreversible changes occurring on larger variations of pH, probably due to some denaturation of the protein.

Hydrazones are well known to undergo hydrazone–azo tautomerism (Krueger, 1975), the equilibrium between the forms being strongly influenced by the nature of the molecule, by the solvent and by synergic effects. A similar tautomerism may occur with hydrazido and hydrazino derivatives of PQQ. This is shown in Scheme 1, together with that for PLP hydrazones. The apparent similarity of the chromophores may explain the similarity of optical properties between BSAO and PLP derivatives with hydrazides and hydrazines. The tautomers of the Schiff bases of PLP with natural amino acids also show analogous spectroscopic properties and little sensitivity to the nature of the amino acid side chain (Casella & Gullotti, 1983). The latter property was explained with localization of the electronic transition in the azomethinic group. It appears that a similar explanation may hold for the insensitivity of BSAO absorptions of the hydrazide nature. Moreover the bands of hydrazine and benzylhydrazine derivatives of BSAO also fall in the same wavelength range, whereas phenylhydrazine, 4-chlorophenylhydrazine, 2,4-dinitrophenylhydrazine and 9-acridinohydrazine derivatives have substantially red-shifted absorption, the shift and intensity of the band increasing with the complexity of the molecule (Table 2). In the latter compounds formation of the azo tautomer leads to conjugation of the aromatic rings with the PQQ π -electron system via the azo group, giving rise to a bathochromic shift and increased intensity of the bands. The azo tautomers involve a reduced hydroquinonic form of the PQQ moiety (Scheme 1), leading to disappearance of BSAO absorption at 480 nm, which is very probably an n – π^* transition of the quinonic group, as reported for polycyclic quinones with absorptions in the same wavelength region (Murrell, 1963). The less pronounced decrease of the 480 nm band in benzhydrazides and semicarbazide may indicate a shift of the equilibrium towards the hydrazone form.

The reversible steps of the process of binding of the

Scheme 1. Tautomerism of hydrazone derivatives of PQQ and of PLP

hydrazides are probably related to the formation of the hydrazone. This is a reversible reaction, as shown by the behaviour of the acetone derivative of 4-(1-pyrryl)-phenylacetic hydrazide, where the acetone moiety is displaced by the protein carbonyl group. In subsequent steps the adduct may become stabilized through hydrogen-bonding, either within the PQQ environment, as shown in Scheme 1, or by interaction with other protein residues. The presence of many equilibrium forms, of which those of Scheme 1 are only an example, is shown by the shift of the optical absorption maximum of 3-(1-pyrryl)phenylacetic hydrazide and by the detection of several intermediates in the catalytic reaction of amine oxidases with substrates (Pettersson, 1985; Bellelli *et al.*, 1985). On the other hand the high stability ('irreversibility') of the phenylhydrazine and analogous derivatives is to be related to the stabilizing effect of the delocalized π -electron system in the azo form.

3-(1-Pyrryl)phenylacetic hydrazide and other hydrazides modify the copper e.p.r. spectrum (Fig. 2) in a way comparable with that of phenylhydrazine either in the native (Mondovi *et al.*, 1986) or *NN*-diethyldithiocarbamate-treated BSAO (Morpurgo *et al.*, 1987). The c.d. spectra reported in Fig. 3 show that *NN*-diethyldithiocarbamate binding to copper modifies the spectrum of the hydrazide by decreasing the intensity of the 395 nm Cotton effect and increasing that at about 340 nm. The latter modifications are reminiscent of the changes produced by pH (Table 1) and are similarly interpreted as being due to a shift of the equilibrium between different tautomers. The possibility of affecting such equilibria through reactions occurring at the metal site, as well as through protonation of some group at the protein catalytic site, may explain the higher reactivity of PQQ in the protein matrix than in the free state. Therefore it is not possible to identify the cofactor as PQQ or PLP on the basis of the reactivity, which may be substantially affected by the proteic matrix and the presence of the metal ions. Removal of copper modifies the reactivity of the cofactor with phenylhydrazine, making the reaction rate lower and decreasing the affinity constant by two orders of magnitude (Suzuki *et al.*, 1986). Copper-bound

NN-diethyldithiocarbamate also decreases the rate of reaction with phenylhydrazine. These effects, which had previously been attributed to conformational changes (Morpurgo *et al.*, 1987), may in fact originate from shifts of tautomeric equilibria through electronic effects.

The above results are in line with the findings reported by Farnum & Klinman (1986) showing that the pH-dependent process of amine oxidation follows a definite, substrate-dependent, stereochemical path. A relatively small change of the acyl residue of the hydrazide, from benzoic acid or phenylacetic acid, considerably decreased the rate of reaction with BSAO. The respective c.d. spectra were more affected than were the optical ones, suggesting a stereochemical effect. An analogous effect was produced by pH changes on c.d. and optical spectra.

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