Bovine Serum Amine Oxidase: Half-Site Reactivity with Phenylhydrazine, Semicarbazide, and Aromatic Hydrazides†

Laura Morpurgo,*,‡ Enzo Agostinelli,‡ Bruno Mondovi,‡ Luciana Avigliano,§ Romano Silvestri,∥ Giorgio Stefancich,⊥ and Marino Artico∥

Centro di Biologia Molecolare del CNR e Dipartimento di Scienze Biochimiche A. Rossi-Fanelli, Università di Roma La Sapienza, 00185 Roma, Italy, Dipartimento di Scienze e Tecnologie Biomediche e Biometria, Università dell'Aquila, 67100 L'Aquila, Italy, Dipartimento di Studi Farmaceutici, Università di Roma La Sapienza, 00185 Roma, Italy, and Dipartimento di Scienze Farmaceutiche, Università di Trieste, 34127 Trieste, Italy

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ABSTRACT: Aromatic hydrazides of the general formula $NH_2NHCO(CH_2)_nC_6H_4R$ were covalently bound by bovine serum amine oxidase (BSAO), giving rise to optical and CD absorptions at 350–400 nm. Benzohydrazides ($n = 0$) reacted slowly, in the ratio of one per dimeric protein molecule, like semicarbazide. Phenylacetohydrazides ($n = 1$) and phenylpropionic hydrazides ($n = 2$) reacted instead in the ratio of two per dimer, one molecule at a much faster rate than the other. The fast reaction correlated with the loss of enzymatic activity. The contribution to the optical absorbance of either molecule was identical, but only the first one produced a CD band, the wavelength and sign of which were determined by the number n of methylene groups in the hydrazide. In $n = 1$ and $n = 2$ compounds, the reaction was faster as the R substituent became more hydrophobic (triazolyl < imidazolyl < phenyl), suggesting a specific interaction with the protein matrix. Phenylhydrazine was found to react with the native enzyme in the ratio of only one per protein dimer. However, one phenylhydrazine was also slowly bound by most 1:1 enzyme–hydrazide adducts, with the formation of ternary derivatives. Phenylhydrazine formed the usual intense band at 447 nm with $n = 1$ and $n = 2$ hydrazide–BSAO adducts and a weaker, blue-shifted band with the adducts of semicarbazide and of some $n = 0$ hydrazides. In both cases, the hydrazide absorption band was unaffected. Competition was observed with other benzohydrazides and with the second molecule of $n = 1$ compounds. A half-site mechanism appears to be operative, the second site being always less reactive than the first. Reactivity and adduct conformation were also affected by *N,N*-diethyldithiocarbamate, a powerful enzyme inhibitor that binds copper.

Bovine serum amine oxidase (BSAO)[1] (EC 1.4.3.6) is a copper-containing enzyme active on primary amines, which are oxidized by oxygen to the corresponding aldehyde with formation of hydrogen peroxide and ammonium ions. It is composed of two subunits of similar M_r and contains two magnetically independent Cu(II) ions and a covalently bound organic cofactor, which is sensitive against reagents which bind carbonyl groups such as semicarbazide and hydrazines (Pettersson, 1985). The cofactor was originally identified as pyrroloquinoline quinone (PQQ) (Ameyama et al., 1984; Lobenstein-Verbeek et al., 1984) and, more recently, as 6-hydroxy-DOPA (TOPA) quinone covalently bound to the polypeptide chain of the protein (Janes et al., 1990). Janes and Klinman (1991) have also reported evidence for the presence of one cofactor per protein subunit, each reacting independently of the other one. This stoichiometry is based on titrations with substrate and with the inhibitor phenylhydrazine. Only a single cofactor per dimeric enzyme was previously found by other research groups using similar techniques. One mole of aldehyde per dimer was obtained on reacting amine oxidase from pig plasma with the substrate benzylamine under anaerobic conditions (Lindström & Pettersson, 1978) and BSAO with the pseudosubstrate benzylhydrazine under aerobic conditions (Morpurgo et al., 1989). Spectroscopic and activity titrations with the inhibitor phenylhydrazine indicated that a single carbonyl is available in BSAO (Rinaldi et al., 1983; Suzuki et al., 1983) and in the pig plasma enzyme (Lindström & Pettersson, 1978; Falk, 1983). Reaction of BSAO with 1 mol of inhibitor was also found using either phenylhydrazine (Morpurgo et al., 1987) or other hydrazines and hydrazides (Morpurgo et al., 1988). One hydrazine, namely 2-hydrazinopyridine, was, however, recently reported to bind the pig plasma enzyme in the ratio of two per dimer, in a biphasic reaction along which the enzyme activity was already abolished by the first, faster binding molecule (Collison et al., 1989). The difference from phenylhydrazine was explained by the authors as due to the greater stability of the new reagent, while Janes and Klinman (1991) attributed their result to the greater purity of the protein samples employed. Half-site reactivity was suggested by Collison et al. (1989) and was excluded by Janes and Klinman (1991).

Some of the aromatic hydrazides, quoted above to react in the ratio of one per BSAO dimer (Morpurgo et al., 1988), were highly specific inhibitors of this enzyme and were poorer inhibitors of other amine oxidases, either those containing copper or those containing FAD (Artico et al., 1988). The study of

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* Author to whom correspondence should be addressed.

‡ Centro di Biologia Molecolare del CNR e Dipartimento di Scienze Biochimiche A. Rossi-Fanelli, Università di Roma La Sapienza.

§ Dipartimento di Scienze e Tecnologie Biomediche e Biometria, Universita dell'Aquila.

∥ Dipartimento di Studi Farmaceutici, Università di Roma La Sapienza.

⊥ Dipartimento di Scienze Farmaceutiche, Università di Trieste.

[1] Abbreviations: BSAO, bovine serum amine oxidase; DDC, *N,N*-diethyldithiocarbamate.

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a larger number of new hydrazido derivatives, which were subsequently synthesized for a better understanding of the reasons of the inhibitor's specificity (Artico et al., 1992), showed that the length of the $-(CH_2)_n-$ aliphatic chain (Figure 1) connecting the hydrazido group and the aromatic moiety of the molecule was of paramount importance, since a different behavior was obtained for $n = 0$, 1, and 2, respectively. In the medium sized $n = 1$ molecules, the reactivity was higher than in the two other groups. This property, and the fact that some unspecific inhibition ability persisted in molecules deprived of the hydrazido function, led to the conclusion that the specificity of these inhibitors is due to the concerted effect of the hydrazide covalent bond and of a hydrophobic interaction between the aromatic group and the protein environment (Artico et al., 1992).

In the present paper, we report a spectroscopic study of the reaction of BSAO with some of the new inhibitors, with special attention to the stoichiometry of binding, in view of the recent results of Collison et al. (1989) and of Janes and Klinman (1991).

Materials and Methods

BSAO was purified by the method of Turini et al. (1982), with the addition of two more purification steps on Q-Sepharose columns, pH 8.0 and pH 6.0, as suggested by Janes et al. (1990). A single band was obtained on SDS gel electrophoresis. The enzymatic activity was measured spectrophotometrically at 25 °C by monitoring the oxidation of benzylamine to benzaldehyde at 250 nm ($\epsilon = 12\,500$ M^{-1} cm^{-1}) (Tabor et al., 1954). The specific activity of the samples used was 0.3–0.4 IU/mg, with IU (international unit) defined as micromoles of oxidized per minute. The protein concentration was measured from the 280-nm absorbance, using an absorption coefficient of 1.74 L g^{-1} cm^{-1} (Suzuki et al., 1983) and $M_r = 180\,000$ (Turini et al., 1982).

Absorption coefficient values vary in the literature from 1.3 L g^{-1} cm^{-1} (Turini et al., 1982) to 2.08 L g^{-1} cm^{-1} (Zeedan et al., 1980), and M_r values vary from 170 000 (Yasunobu et al., 1976) to 190 000 (Suzuki et al., 1983). A redetermination of the absorption coefficient of the protein at 280 nm by the Bio-Rad procedure using bovine serum albumin or ascorbate oxidase from zucchini peelings as a standard yielded a value of 1.7 ± 0.2 L g^{-1} cm^{-1}. A somewhat higher value of 2.0 ± 0.4 L g^{-1} cm^{-1} was obtained using the biuret procedure. The value of 1.3 L g^{-1} cm^{-1} (Turini et al., 1982) was obtained by the dry weight method. This is apparently not well suited to BSAO, a scarcely water soluble protein, which tends to precipitate when the buffer is dialyzed away. The M_r of BSAO subunits was recently reexaminated by SDS/mercaptoethanol disc gel electrophoresis (M. T. Graziani, unpublished results), resulting in a value close to 90 000, in agreement with the 180 000 value previously obtained by sedimentation–diffusion (Turini et al., 1982). Using this M_r value, a best fit to a copper content of 2.0 ± 0.1 Cu ions per dimer is obtained with an extinction coefficient close to 1.7 L g^{-1} cm^{-1}. This justifies the choice of 1.74 L g^{-1} cm^{-1} made many years ago (Morpurgo et al., 1987, 1988, 1989). Consistent results for copper analyses were obtained with the biquinolyl method (Brumby & Massey, 1967), double integration of the EPR spectra, and atomic absorption spectrophotometry on a Perkin-Elmer 3030 apparatus equipped with an HGA-400 graphite furnace (Morpurgo et al., 1987). All experiments were carried out in air at 25 °C in 0.1 M potassium phosphate buffer, pH 7.2. Optical spectra were recorded on a λ-9 Perkin-Elmer spectrophotometer; CD spectra were recorded on a Jasco 500 spectropolarimeter.

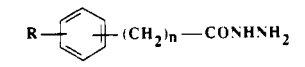

FIGURE 1: General formula of hydrazides.

Table I: Phenylhydrazine Titratable Carbonyl and Copper Content of BSAO as a Function of the Sample Specific Activity[a]

sp act. (IU/mg)	active carbonyl (mol/dimer)	copper (ions/dimer)
0.40 (0.48)	0.98 (1.45)	2.02
0.36 (0.43)	1.02 (1.51)	1.95
0.32 (0.38)	1.07 (1.58)	1.95
0.30 (0.36)	0.91 (1.35)	1.80
0.29 (0.35)	0.87 (1.29)	1.71
0.24 (0.29)	0.86 (1.27)	1.98
0.24 (0.29)	0.79 (1.17)	1.67

[a] The values obtained are based on a 280-nm absorbance of E = 1.74 L g^{-1} cm^{-1}, $M_r = 180\,000$, and a 447-nm absorbance of the BSAO–phenylhydrazine adduct of $\epsilon = 41\,500$ M^{-1} cm^{-1}. The values in parentheses are based on the parameters used by Janes and Klinman (1991), namely, a 280-nm absorbance of E = 2.08 L g^{-1} cm^{-1}, $M_r = 170\,000$, and a 447-nm absorbance of $\epsilon = 32\,400$ M^{-1} cm^{-1}.

Preparation and physical properties of the hydrazides used in the present work are reported elsewhere (Artico et al., 1992) and their general formula is shown in Figure 1. Benzaldehyde, semicarbazide, and acetohydrazide were commercial products from Merck or Fluka AG. Hydrazides carrying a phenyl substituent were dissolved in methanol. In the reaction mixture, methanol did not exceed 0.5% in volume. Phenylhydrazine (a Merck reagent) produced identical results as an ethanol recrystallized sample, a kind gift of Dr. G. Floris (University of Cagliari, Italy).

Results

Reaction with Phenylhydrazine. Phenylhydrazine is an unstable substance which deteriorates in solution (Robertson et al., 1989) and also deteriorates very slowly in the solid state. Titrations of BSAO with pure (ethanol recrystallized) phenylhydrazine produced an increase of absorbance at 447 nm up to a ratio of 1.0–1.1 mol:protein dimer. The value of $\epsilon = 41\,500$ M^{-1} cm^{-1} or $\Delta\epsilon = 38\,000$ M^{-1} cm^{-1} was determined from difference spectra, taking native protein as the baseline. In Table I are reported the results obtained for samples of different specific activity together with the values of the copper content. A single phenylhydrazine and two copper ions per BSAO dimer were found when the specific activity was ≥0.32 IU/mg of enzyme. Less active samples gave a lower figure for phenylhydrazine reactive carbonyl and copper content, but the 1:2 ratio between the two cofactors was conserved. The following reaction with phenylhydrazine is routinely used in our laboratory as a sensitive and easy test of protein purity (Morpurgo et al., 1987, 1988, 1989). BSAO solutions are saturated with phenylhydrazine, and the absorbance at 447 nm is read after 15 min. The chromophore is stable in air for days at 5 °C.

The reactivity of two phenylhydrazines per BSAO dimer reported by Janes and Klinman (1991) was based on an extrapolation of data showing an increasing trend of the phenylhydrazine:BSAO ratio as a function of specific activity. The samples used in this study were considered to be more highly purified than before. The maximum ratio of phenylhydrazine bound per protein dimer actually measured was 1.6. The discrepancy with our data is considerable and only to a limited extent imputable to the use of a different M_r and molar extinction coefficient at 280 nm, respectively. Such differences are relatively small, −6% and +20%, respectively, as compared to the values used in our own studies, and moreover, they

Table II: Spectral Properties of BSAO Adducts with Benzohydrazides (from Difference Spectra with Respect to the Native Protein)

	near UV		CD	
inhibitor	λ (nm)	Δε (M^{-1} cm^{-1})	λ (nm)	Δε (M^{-1} cm^{-1})
semicarbazide	360	18 700	365	+28.1
acetohydrazide	358	26 300		
benzohydrazide substituents				
H	355	22 800	360	+36.3
o-imidazol-1-yl				
p-imidazol-1-yl	352	22 100	360	+23.6
o-(imidazol-1-ylmethyl)				
m-(imidazol-1-ylmethyl)	352	22 500	359	+33.9
p-(imidazol-1-ylmethyl)	352	22 500	358	+33.2
p-pyrazol-1-yl	360	17 000	360	+12.0
p-triazol-1-yl	359	22 000	359	+16.2
p-phenyl	359	17 100	360	+17.3

compensate in the calculation of the ratio, leaving an overall difference of only 13%. A larger source of discrepancy with our results is the titration with phenylhydrazine, from which a nearly 30% lower molar extinction coefficient for the BSAO adduct was estimated by Janes and Klinman (1991). This value is independent of the protein purity, which was comparable in the two laboratories since the use by Janes and Klinman of a higher molar extinction coefficient at 280 nm resulted in about 20% overestimation of the activity with respect to our values. On the contrary, the low activity value of 0.23 IU/mg of Turini et al. (1982) was the result of a 25% underestimation of the extinction coefficient with respect to our value. The effect of the physical parameters used by Janes and Klinman and by ourselves in the analysis of the data is shown in Table I, which reports specific activity and phenylhydrazine:dimer ratios. In our case, the ratio levels off at 1.0 phenylhydrazine/dimer, and in the case of Janes and Klinman, at 1.5.

Reactions of BSAO with Hydrazides. The reaction of BSAO with hydrazides forms an adduct characterized by an intense absorption band at 350–360 nm and by a CD band at 360–400 nm. Differences, however, were found for the individual adducts, depending on the number of methylene groups present in the hydrazide molecule.

Benzohydrazides (n = 0, Table II) as well as semicarbazide and acetohydrazide show a high intensity optical band (Δε = 17 000–26 000 M^{-1} cm^{-1}) at 352–360 nm and a positive Cotton effect at about the same wavelength. The reaction of these compounds with 10 μM BSAO dimer in a 1:1 ratio required 1–2 h for the attainment of a constant absorption value. Within this time, only the intensity of the spectra changed, not the shape or position of the band maximum. With excess hydrazide the reaction was faster, but an identical maximum intensity was reached. Ortho-substituted benzohydrazides were completely unreactive, while acetohydrazide reacted at a slower rate than the others. The unsubstituted benzohydrazide adduct was previously incorrectly reported to display a negative Cotton effect at 400 nm (Morpurgo et al., 1988). It shows a positive band at 360 nm as do the other adducts of the group.

Phenylacetohydrazides (n = 1, upper part of Table III), in a 1:1 ratio with BSAO, formed a band at 360 nm in a few minutes that was substantially less intense than that of benzohydrazides and a negative CD band. The optical difference spectra of typical adducts of benzohydrazide and phenylacetohydrazide, at a 1:1 ratio with BSAO, are compared in Figure 2A and the corresponding CD spectra are in Figure 2B. The shift of the CD transition to a longer wavelength in phenylacetohydrazide adducts suggests that the broad optical band is the envelope of at least two transitions, with only one having optical activity. Excess phenylacetohydrazide gave rise to a biphasic process in which the fast initial formation of the band was followed by a very slow further increase that leveled off after at least 24 h. The band produced in the slow step had the same shape and wavelength maximum as the first one. The intensity after 24 h was approximately twice that obtained at 1:1 inhibitor:BSAO ratio (Table III). The negative Cotton effect near 400 nm that was produced within 1 min, concomitant with the fast phase, showed a slight decrease at the end of the slow one. The formation of optical and CD bands and the loss of enzymatic activity along a titration with *p*-phenylphenylacetohydrazide is displayed in Figure 3 as a function of the [inhibitor]:[BSAO dimer] ratio. The optical density was monitored at 1 min and 24 h, at the end of the fast and slow phases, respectively. Very small differences between each couple of points were found at less than stoichiometric concentrations of the inhibitor. It is apparent that 1 molecule of inhibitor:dimer was bound in the fast process, with loss of the enzymatic activity, while 2 molecules were bound after 24 h.

The third group of compounds, that of phenylpropionic hydrazides (n = 2, lower section of Table III), only differ from phenylacetohydrazides in that the CD spectrum showed a positive band at 370 nm, which probably corresponds to a different component of the similar optical absorption band. The reactivity was similar, though a small difference appeared

Table III: Spectral Properties of the BSAO Adducts with Phenylacetic and Phenylpropionic Hydrazides (from Difference Spectra with Respect to the Native Protein)

	near UV[a]			CD[a]		
		Δε (M^{-1} cm^{-1})			Δε (M^{-1} cm^{-1})	
substituent	λ (nm)	A*	B*	λ (nm)	A*	B*
Phenylacetic Hydrazide Substituents						
p-phenyl	358	7900	16 800	398	−15.4	−19.5
p-imidazol-1-yl	365	7900	17 000	400	−13.5	−26.3
p-triazol-1-yl	358	9400	16 900	400	−10.9	−15.3
o-(imidazol-1-ylmethyl)	352		25 000	359		+19.0
p-(imidazol-1-ylmethyl)	363	11 900	19 000	399	−23.8	−27.0
p-pyridine-2-yl	354	11 100	17 500	400	−11.5	−16.7
p-pyrazol-1-yl	352	10 200	21 100	400	−8.5	−14.0
Phenylpropionic Hydrazide Substituents						
p-imidazol-1-yl	352	9 500	18 000	365	+13.0	+16.2
p-pyrazol-1-yl	352	10 500	18 000	370	+15.2	+15.2
p-triazol-1-yl	354	10 600	19 000	365	+14.4	+15.9
p-phenyl	363	10 100	18 300	378	+18.3	+18.6

[a] An asterisk indicates that the values were obtained at 1:1 hydrazide:BSAO dimer ratio after a 1-h incubation (A) or with a 10-fold hydrazide excess after 24 h incubation (B).

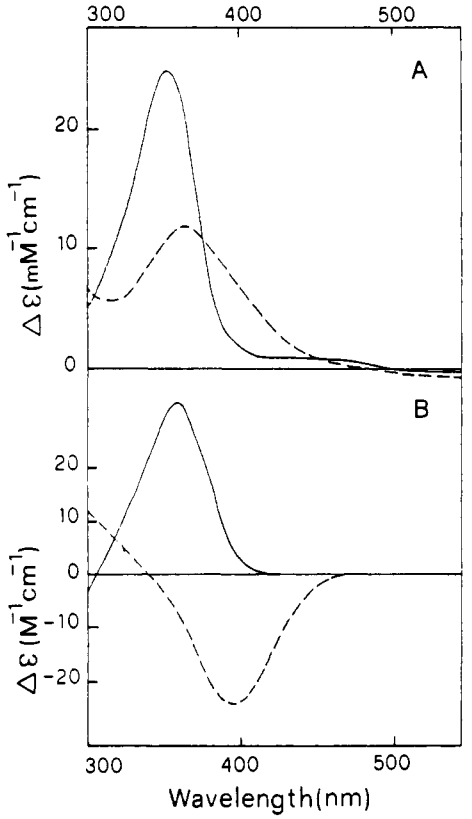

FIGURE 2: Optical absorption spectra (A) and CD spectra (B) of BSAO adducts with stoichiometric hydrazides: *p*-(imidazol-1-ylmethyl)benzohydrazide after 60 min of incubation (solid line) and *p*-(imidazol-1-ylmethyl)phenylacetohydrazide after 10 min of incubation (dashed line). Difference spectra are with the native protein (10 μM) as reference.

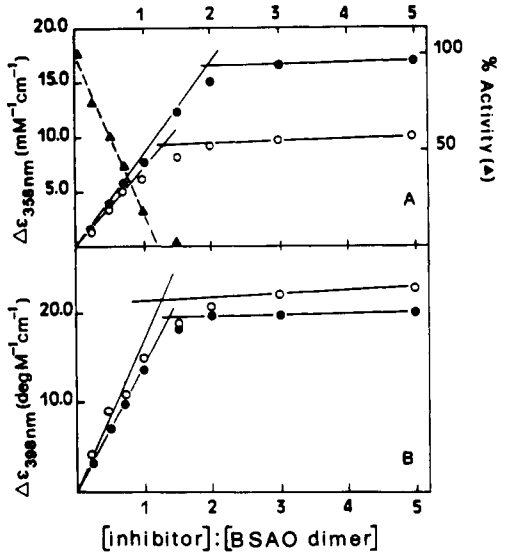

FIGURE 3: BSAO–*p*-phenylphenylacetohydrazide adducts: correlation between inhibition and spectroscopic properties. A 20 μM concentration of BSAO was used. Its absorbance and ellipticity in the absence of inhibitor at the indicated wavelengths (see Table III) were used as the ordinate zero. Optical (A) and CD spectra (B) were recorded on the same solution after 1 and 5 min, respectively (○), and after 24 h, respectively (●). Activity measurements (▲) were performed immediately after the optical spectrum and were recorded on the solution diluted 1:50. CD spectra and activity were unchanged from 1 to at least 30 min.

at a closer inspection. Figure 4 shows the reaction, at 1:1 ratio with BSAO, of two phenylacetohydrazides (solid lines) and two phenylpropionic hydrazides (dashed lines), in which R was a phenyl group (two upper lines) or a triazolyl group (two

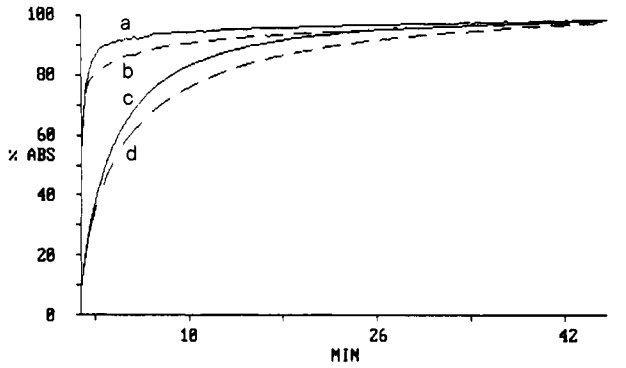

FIGURE 4: Time course of the reaction of BSAO with stoichiometric hydrazides: phenylacetic (solid lines) and phenylpropionic hydrazides (dashed lines), in which R was *p*-phenyl (curves a and b) or *p*-triazolyl (curves c and d). A 10 μM concentration of native BSAO was used, the absorbance of which at 352 nm was taken as the ordinate zero.

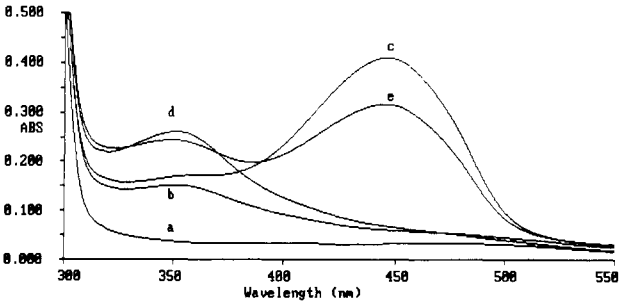

FIGURE 5: Reaction of phenylhydrazine with BSAO pyridin-2-ylphenylacetohydrazone. A 10 μM concentration of BSAO (a) was incubated 24 h with stoichiometric hydrazide (b) plus 24 h with stoichiometric phenylhydrazine (c) or 24 h with a 2-fold hydrazide excess (d) plus 24 h with a 2-fold phenylhydrazine excess (e).

lower lines). Imidazolyl derivatives, falling in between the two groups of lines, were omitted for the sake of clarity. Phenylpropionic hydrazides reacted more slowly than phenylacetohydrazides, but they preserved an identical dependence on the nature of the substituent. The reaction of these compounds was also biphasic, leading to binding of two molecules per BSAO dimer when an excess was used.

Ternary Adducts: BSAO–Hydrazide–Phenylhydrazine. In a previous study (Morpurgo et al., 1988) competition was believed to occur between phenylhydrazine and phenylacetohydrazides since the CD spectra of the hydrazide adducts were decreased upon addition of phenylhydrazine during the time period when the optical and CD bands of the phenylhydrazine adduct were formed. When it became clear that two molecules of phenylacetohydrazide could be bound without a strict correlation of optical and CD spectra since only one molecule produced a CD band, a reinvestigation of the phenylhydrazine reaction was begun. It was again found that the band from phenylhydrazine at 447 nm could be fully developed in the presence of most BSAO–phenylacetohydrazide adducts if a sufficiently long time was allowed for the reaction to occur, while the CD band of the hydrazide adduct was suppressed. Figure 5 shows the spectra of BSAO first treated with pyridin-2-ylphenylacetohydrazide, either stoichiometric (curve b) or in a 2-fold excess (curve d), and then with an identical amount of phenylhydrazine (curves c and e). The two experiments differ in that the hydrazide band at 354 nm did not decrease in the 1:1 compound, although the corresponding CD band disappeared. Some decrease, however, occurred in the solution containing excess hydrazide (curve e). These results show that phenylhydrazine was additionally bound by the 1:1 compound, while it displaced one of the two bound hydrazide molecules from the 2:1 derivative. An identical ternary adduct

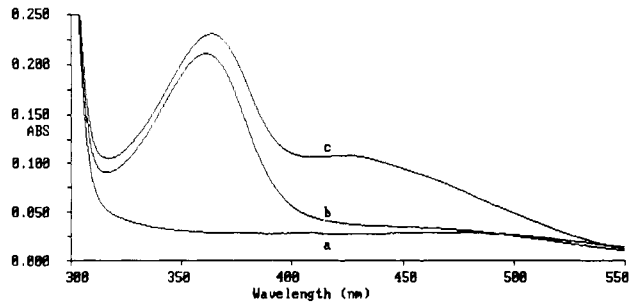

FIGURE 6: Reaction of phenylhydrazine with BSAO–semicarbazone. A 10 μM concentration of BSAO (a) was incubated for 2 h with a 2-fold excess of semicarbazide (b) plus 90 min with stoichiometric phenylhydrazine (c).

was eventually formed in both experiments. The 1:1 adducts of phenylpropionic hydrazides were able to react like the corresponding phenylacetic derivatives, while 2:1 adducts were stable against phenylhydrazine.

The reactions of benzohydrazide adducts with phenylhyrazine were more complicated. Phenylhydrazine displaced the hydrazide from the adducts of phenyl and triazolyl derivatives, as shown by the decrease of both optical and CD bands of the adduct upon formation of the 447-nm phenylhydrazine absorption. Optical and CD spectra of *p*- and *m*-(imidazol-1-ylmethyl)benzohydrazide and of the unsubstituted benzohydrazide adducts were unaffected by phenylhydrazine while a band of low intensity ($\Delta\epsilon$ = 8000–10 000 M^{-1} cm^{-1}) was formed around 430 nm after prolonged incubation. With the BSAO–semicarbazide adduct the band occurred at 420 nm and the reaction was faster. The latter spectra are reported in Figure 6. After 90 min of incubation with stoichiometric phenylhydrazine, approximately 0.7 mol of the reagent was consumed, in spite of the relatively low intensity of the new band. The above value was obtained by titrating unreacted phenylhydrazine with native BSAO. Titration of the stock solution of phenylhydrazine with native BSAO showed that no appreciable decomposition had occurred within the incubation time. Overnight incubation increased the intensity of the band by about 30%, but small side effects imputable to phenylhydrazine autoxidation were also observed. It is possible to conclude that also in this case a ternary adduct was formed, in which phenylhydrazine displayed an absorption band at a shorter wavelength and of lower intensity than that formed with native BSAO. The semicarbazone CD band at 360 nm was replaced by a band at 420 nm.

Reactions in the Presence of Copper Binding Enzyme Inhibitors. Hydrazides were also added to the protein preincubated with 5.0 mM *N*,*N*-diethyldithiocarbamate (DDC), an enzyme inhibitor that tightly binds copper, as previously done with phenylhydrazine (Morpurgo et al., 1987). The reaction was in every case much slower than that of the native protein. The optical and CD spectra of benzohydrazide adducts were unaffected by the presence of the chelator, while the optical spectra of the phenylacetohydrazide adducts were shifted to lower wavelength and their intensity increased, approaching that of the benzohydrazide adducts. The CD spectra were also shifted to a lower wavelength in the range of that of the benzohydrazide adducts, but the negative sign of the band persisted as found in the reaction of the BSAO–pyrrolyl derivative (Morpurgo et al., 1988). The 0.1 M azide, a weaker enzyme inhibitor that reversibly binds copper, had no effect on these reactions.

Discussion

Stoichiometry of Carbonyl Groups. A number of laboratories reported that BSAO and the similar enzyme from pig plasma reacted with phenylhydrazine in the ratio of one per protein dimer. Recent reports (Klinman et al., 1989; Janes et al., 1990; Janes & Klinman, 1991) stated that the ratio is two per BSAO dimer, explaining this result on the basis of the higher specific activity of the protein preparations they used. The data of Table I show that our BSAO samples have a comparable specific activity and yet they bind only one phenylhydrazine per BSAO dimer. The differences in the BSAO M_r and extinction coefficient at 280 nm used (see Table I and the Results section) are not large and in part compensate for each other in the calculations. The lower value we report for the phenylhydrazine:BSAO ratio is therefore essentially ascribed to a smaller amount of phenylhydrazine being used in the titration, which is reflected by the higher molar extinction coefficient of the product at 447 nm. In addition, we found a constant 1:1 ratio between phenylhydrazine and BSAO dimer in samples of specific activity >0.32 and a constant 2:1 ratio between copper content and phenylhydrazine bound in all samples. It seems reasonable to conclude that these ratios and the parameters we used in the calculations are the correct ones.

Further proof that 1:1 reactivity with phenylhydrazine is not imputable to poor sample activity is provided by the ability of the same sample to react in either 1:1 or 2:1 inhibitor:dimer ratio depending on the nature of the inhibitor. Phenylhydrazine and benzohydrazides each reacted in 1:1 ratio, while phenylacetic and phenylpropionic hydrazides reacted in a 2:1 ratio. Moreover, the same sample that was only able to bind a single phenylhydrazine in the native state could form ternary adducts by binding one phenylhydrazine after one hydrazide was already bound. In the latter case, the reaction with phenylhydrazine was as slow as the binding of a second hydrazide, while it is fast with native BSAO. These simple reactions were produced by a large number of different stable compounds.

Hydrophobic Interactions and Conformation of the Adducts. Phenylacetohydrazides and phenylpropionic hydrazides were found to be efficient inhibitors of BSAO and poor inhibitors of either copper-containing diamine oxidases or FAD-dependent monoamine oxidases (Artico et al., 1988, 1992). The specificity was found to originate from interactions between the aromatic substituent in the phenyl ring and a hydrophobic site near the carbonyl. The fastest reactions were obtained with para-substituted phenylacetohydrazides, which therefore appear to have the best shape to fit the protein active site, forming a covalent bond with the hydrazidic group and a hydrophobic interaction with the aromatic portion of the molecule. These conclusions are confirmed by the spectroscopic data reported in Tables II and III. They show in fact that homologous compounds, only differing in the length of the aliphatic chain, formed adducts with a different conformation that was the same for all compounds with an identical number of methylene groups. The time of reaction was also different in the three groups so that $n = 1 < n = 2 <<< n = 0$. Within the two more reactive groups, the presence and the nature of the R substituent also influenced the reaction time, which tended to increase in the presence of the more hydrophobic substituent, phenyl > imidazolyl > triazolyl, in line with a hydrophobic interaction occurring in the proximity of the active site (Artico et al., 1992). The influence of the molecular shape is apparent in the case of *o*-(imidazol-1-ylmethyl)phenylacetohydrazide, which contains a methylene spacer between the phenyl and the R ring. This compound showed some reactivity unlike other ortho-substituted hydra-

zides, but the adduct was slowly formed and it was forced into the conformation peculiar to benzohydrazide instead of phenylacetohydrazide adducts. Such conformations were previously tentatively assigned as tautomeric hydrazone and azo forms, respectively (Morpurgo et al., 1988). Whatever the real nature, this is regulated by steric factors connected with the number of methylene groups. The chromophore appears to be localized on the cofactor, being quite insensitive to the nature and even to the presence of the aromatic portion of the inhibitor. We would like to recall here that hydrazine adducts behaved differently, since their optical band was much more sensitive to the nature of the molecule. When the π-system of the cofactor could conjugate with that of the hydrazine derivative through the –N=N– linkage, the optical band was shifted to longer wavelength, was more intense, and was sensitive to substituents in the ring (Morpurgo et al., 1988). The formation of the azo bond implies the shift of two electrons toward the cofactor that assumes an electronic configuration close to that of the reduced hydroquinone species.

Half-Site Mechanism. The results discussed above lead to the conclusion that BSAO contains two cofactors per protein dimer but has a half-site reactivity, as previously suggested to occur in the pig plasma enzyme (Collison et al., 1989), since the catalytic activity was abolished and the reactivity of the second cofactor decreased by the binding of only one inhibitor molecule per protein dimer. Molecules which "reduce" the cofactor, such as substrates and hydrazines, appear to cause a conformational change of the protein that completely inhibits the reaction of the second cofactor, while hydrazides only slow down the binding.

A further difference was observed between benzohydrazides on one side and phenylacetic and phenylpropionic hydrazides on the other. Benzohydrazide adducts were only able to interact with phenylhydrazine, the most active ligand, that either bound in place of the hydrazide or formed a ternary adduct in which it was bound in a distorted conformation, as shown by the modified optical spectrum (Figure 6). The other ones could bind in 2:1 ratio and allowed binding of phenylhydrazine in the usual way. The more rigid, slow reacting benzohydrazides appear to require a larger conformational change of the BSAO dimer that impairs the reaction of the second site, while the more flexible phenylacetic and phenylpropionic hydrazides can bind with less dramatic steric effects. Similar reasons, rather than a different stability of the reagent, may explain why pig plasma amine oxidase could bind two molecules of 2-hydrazinopyridine, but only one of phenylhydrazine (Collison et al., 1989). The absorption band of the former derivative has a shorter wavelength and much lower intensity (ϵ = 15 400 M^{-1} cm^{-1} at 415 nm; Collison et al., 1989) than that of phenylhydrazine, indicative of a different conformation. Phenylhydrazine forms a similar band on reacting with the semicarbazide–BSAO adduct. The difference may be due to a hydrazone–azo tautomerism (Morpurgo et al., 1988), but alternative conformations are possible. Comparable spectral differences are reported to exist between trans and cis or protonated and neutral forms of azobenzene (Jaffé & Orchin, 1966). The high intensity and long wavelength of the phenylhydrazone absorption band could indicate protonation of the azo group. This usually occurs in strongly acidic medium, but hydrogen bonding to the cofactor hydroxyl in the ortho position may produce analogous spectral effects.

The discussion above implies the presence of two cofactors per dimer, which are capable of binding the inhibitors covalently. The theoretical possibility that the slow reaction involves the second C=O of the same quinonoid cofactor is excluded by the fact that a slowly binding second inhibitor molecule never caused a modification of preexisting optical bands. The implication of a carbonyl from the glucidic component of BSAO seems as well unlikely, as it would form with each inhibitor identical absorption bands as the cofactor.

DDC is an inhibitor that very tightly binds the copper ions in BSAO and slows down, but does not abolish, the mechanism based oxidation of the pseudosubstrate benzylhydrazine (Morpurgo et al., 1989). The optical spectrum of phenylhydrazine bound to the BSAO–DDC adduct (Morpurgo et al., 1987) is similar to that of the ternary adduct with semicarbazide. The reactivity of the BSAO–DDC adduct with hydrazides is also comparable to that of BSAO–hydrazide adducts with a second inhibitor molecule. All sites competent to catalytic activity appear to be connected through the protein backbone so that any change propagates to the others.

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REFERENCES

Ameyama, M., Hayasashi, M., Matsushita, K., Shinagawa, E., & Adachi, O. (1984) *Agric. Biol. Chem. 48*, 561–565.

Artico, M., Corelli, F., Massa, S., Stefancich, G., Avigliano, L., Befani, O., Marcozzi, G., Sabatini, S., & Mondovi, B. (1988) *J. Med. Chem. 31*, 802–806.

Artico, M., Silvestri, R., Stefancich, G., Avigliano, L., Di Giulio, A., Maccarrone, M., Agostinelli, E., Mondovi, B., & Morpurgo, L. (1992) *Eur. J. Med. Chem.* (in press).

Brumby, P. E., & Massey, V. (1967) *Methods Enzymol. 10*, 473–474.

Collison, D., Knowles, P. F., Mabbs, F. E., Rius, F. X., Singh, I., Dooley, D. M., Cotē, C. E., & McGuirl, M. (1989) *Biochem. J. 264*, 663–669.

Falk, M. C. (1983) *Biochemistry 22*, 3740–3745.

Janes, S. M., & Klinman, J. P. (1991) *Biochemistry 30*, 4599–4605.

Janes, S. M., Mu, D., Wemmer, D., Smith, A. J., Kaur, S., Maltby, D., Burlingame, A. L., & Klinman, J. P. (1990) *Science 248*, 981–987.

Jaffē, H. H., & Orchin, M. (1966) in *Theory and Applications of Ultraviolet Spectroscopy*, pp 276–286, John Wiley & Sons, Inc., New York.

Klinman, J. P., Hartmann, C., & Janes, S. M. (1989) in *PQQ and Quinoproteins* (Jongejan, J. A., & Duine, J. A., Eds.) pp 297–305, Kluwer Academic Publishers, Dordrecht.

Lindström, A., & Pettersson, G. (1978) *Eur. J. Biochem. 83*, 131–135.

Lobenstein-Verbeek, C. L., Jongejan, J. A., Frank, J., & Duine (1984) *FEBS Lett. 170*, 305–309.

Morpurgo, L., Agostinelli, E., Befani, O., & Mondovi, B. (1987) *Biochem. J. 248*, 865–870.

Morpurgo, L., Befani, O., Sabatini, S., Mondovi, B., Artico, M., Corelli, F., Massa, S., Stefancich, G., & Avigliano, L. (1988) *Biochem. J. 256*, 565–570.

Morpurgo, L., Agostinelli, E., Muccigrosso, J., Martini, F., Mondovi, B., & Avigliano, L. (1989) *Biochem. J. 260*, 19–25.

Pettersson, G. (1985) in *Structure and Function of Amine Oxidases* (Mondovi, B., Ed.) pp 105–120, CRC Press, Boca Raton, FL.

Rinaldi, A., Floris, G., Sabatini, S., Finazzi-Agro, A., Giartosio, A., Rotilio, G., & Mondovi, B. (1983) *Biochem.*

Biophys. Res. Commun. 115, 841–848.
Robertson, J. G., Kumar, A., Mancewicz, J. A., & Villafranca, J. J. (1989) *J. Biol. Chem. 264*, 19916–19921.
Suzuki, S., Sakurai, T., Nakahara, A., Manabe, T., & Okuyama, T. (1983) *Biochemistry 22*, 1630–1635.
Tabor, C. W., Tabor, H., & Rosenthal, S. M. (1954) *J. Biol. Chem. 208*, 645–661.
Turini, P., Sabatini, S., Befani, O., Chimenti, F., Casanova, C., Riccio, P. L., & Mondovi, B. (1982) *Anal. Biochem. 125*, 294–298.
Yasunobu, K. T., Ishitaki, H., & Minamiura, M. (1976) *Mol. Cell. Biochem. 13*, 3–29.
Zeidan, H., Watanabe, K., Piette, L. H., & Yasunobu, K. T. (1980) *J. Biol. Chem. 255*, 7621–7626.

Analysis of the Fis-Dependent and Fis-Independent Transcription Activation Mechanisms of the *Escherichia coli* Ribosomal RNA P1 Promoter

Martin Zacharias,[‡,§] Hans Ulrich Göringer,[∥] and Rolf Wagner*,[⊥]

Max-Planck-Institut für Molekulare Genetik, Abteilung Wittmann, Ihnestrasse 73, D-1000 Berlin 33, Germany, Seattle Biomedical Research Institute, 4 Nickerson Street, Seattle, Washington 98109-1651, and Institut für Physikalische Biologie, Heinrich-Heine-Universität, Düsseldorf, Universitätsstrasse 1, D-4000 Düsseldorf 1, Germany

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ABSTRACT: The role of the curved DNA sequence upstream to the *Escherichia coli* ribosomal RNA P1 promoter in transcription activation was studied. This sequence region had been shown to activate transcription from P1 in vivo and in vitro and to harbor binding sites for the trans-activating protein Fis. We have constructed a series of linker scanning mutants spanning the region −104 to −47, relative to the transcription start site. DNA fragments carrying the mutations show altered gel electrophoretic mobilities, consistent with reduced DNA bending angles compared to the wild-type sequence. Using gel retardation assays, qualitative as well as quantitative differences in the binding of the trans-activating protein Fis to the mutant DNA fragments could be observed. The effects of the mutations on *rrnB* P1 promoter activation were studied in vivo in *fis*$^+$ and *fis*$^-$ backgrounds. A reduction in the promoter strength for some of the linker mutants correlates with altered Fis binding to two of the known Fis binding sites. Shifting the Fis binding region by half a helical turn, relative to the promoter core sequence, abolishes Fis-mediated activation almost totally, whereas activation is partly restored by a shift of a complete helical turn. For one mutant, which does not show alterations in Fis binding, a decrease in the promoter strength was observed in a *fis*$^-$ strain. From the results, we conclude that two upstream activating mechanisms, one Fis-dependent and one Fis-independent, influence the *rrnB* P1 promoter strength. Sequence determinants for the Fis-independent mechanism are closer to the promoter core region than the Fis binding sites. In addition, the study demonstrates that both the helical geometry and the absolute distance of the UAS region relative to the promoter are crucial for transcription activation.

In bacteria, transcription initiation is known to start from consensus DNA promoter structures (McClure, 1985). In addition to the conserved −35 and −10 core promoter elements, sequences upstream and downstream from the consensus region determine the efficiency of the initiation process (Deuschle et al., 1986). The strong expression of many stable RNA genes (ribosomal RNA and tRNA genes) is known to be dependent on the presence of AT-rich upstream sequences, located between nucleotides −40 and −150, relative to the transcription start site (UAS regions) (Bossi & Smith, 1984; Lamond & Travers, 1983; Bauer et al., 1988; Plaskon & Wartell, 1987). In the case of the *rrnB* ribosomal RNA promoter P1, a sequence region between nucleotides −50 and −88 was identified which stimulates the transcription about 20-fold (Gourse et al., 1986). Although the upstream promoter sequences of stable RNA genes do not show significant primary sequence homology, they share a common high AT content and a peculiar physical property: DNA fragments containing UAS sequences are generally associated with a stable DNA curvature. These DNA conformational distortions can be identified by an altered electrophoretic mobility of the corresponding DNA fragments. Replacing the natural UAS region of the *rrnA* P1 promoter by a curved DNA fragment from the unrelated organism *Crithidia fasciculata*, as shown recently, restores the original activity to 70% (Nachaliel et al., 1989).

Today there are no experimentally justified mechanistic explanations of how the DNA conformation participates in promoter activation, and there is growing evidence that DNA-binding proteins are involved in the activation mechanism.

The *Escherichia coli* Fis protein, originally identified to stimulate phage Mu *gin* and Salmonella *hin* site-specific DNA inversion (Kahmann et al., 1985; Johnson & Simon, 1985), was shown to bind upstream of *rrnB* P1, *tufB*, and *tyrT* promoters. Addition of Fis can activate transcription from these promoters in vitro (Nilsson et al., 1990; Ross et al., 1990). Gel retardation can resolve at least three different Fis–UAS complexes. In the case of the *rrnB* P1, the corresponding three Fis binding sites were characterized by DNase I footprinting experiments. Although in vitro effects of Fis on the transcription from *rrnB* P1 and *tufB* promoters are dramatic under certain conditions, the promoter strength of the above pro-

* To whom correspondence should be addressed.
‡ Max-Planck-Institut für Molekulare Genetik.
§ Present address: Department of Chemistry, University of Houston, Houston, TX 77204-5641.
∥ Seattle Biomedical Research Institute.
⊥ Heinrich-Heine-Universität.

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