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Modulation of Bovine Serum Amine Oxidase Activity by Hydrogen Peroxide

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Bovine serum amine oxidase (BSAO), reduced by excess amine under limited turnover conditions, was over 80% inactivated by H_2O_2 upon oxygen exhaustion. The UV–Vis spectrum and the reduced reactivity with carbonyl reagents showed that the cofactor topaquinone (TPQ) was stabilized in reduced form. The protein large M_r (170 kDa) prevented the identification of modified residues by amino acid analyses. Minor changes of the Cu^{2+} EPR signal and the formation of a radical at $g = 2.001$, with intensity a few percent of that of the Cu^{2+} signal, unaffected by a temperature increase, suggest that Cu^{2+}-bound histidines were not oxidized and the radical was not the Cu^{+}–semiquinolamine in equilibrium with Cu^{2+}–aminoquinol. It may derive from the modification of a conserved residue in proximity of the active site, possibly the tyrosine at hydrogen-bonding distance of TPQ C-4 ionized hydroxyl. The inactivation reaction appears to be a general feature of copper-containing amine oxidases. It may be part of an autoregulatory process *in vivo*, possibly relevant to cell adhesion and redox signaling.

Key Words: **copper-amine oxidase; enzyme inactivation; hydrogen peroxide; radical reaction; topaquinone.**

Bovine serum amine oxidase (BSAO) belongs to the class of Cu^{2+}-containing amine oxidases (EC 1.4.3.6), ubiquitary enzymes which catalyze the oxidative deamination of primary amino groups with two-electron reduction of molecular oxygen (1):

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2.$$

They contain a second cofactor, TPQ, or topaquinone (2), which is produced by the posttranslational oxidation of a tyrosine residue, catalyzed by the same Cu^{2+} (3, 4). The biological functions of these proteins range from the utilization of amine substrates as a source of carbon and nitrogen in procariotes, to lignification and defence in plants, to detoxification from elevated levels of amines and histamine in mammals. New recently described functions include a possible involvement in posttranslational modifications of proteins (5), in elastine maturation (6), in sodium transport (7, 8), and in cellular adhesion (9). An impressive sequence identity with copper-containing amine oxidases was demonstrated for the human kidney amiloride-binding protein, involved in epithelial sodium transport (7, 8), and for the adhesion protein I (VAP-1) (9), involved in lymphocyte–endothelial cell interactions. The amine oxidase catalytic reaction forms H_2O_2, generally considered to be a toxic waste product, but recently revaluated as one of the reactive oxygen species involved in membrane receptor signaling and in the regulation of cellular functions (10).

This paper reports an investigation of the effects of limited turnover conditions on BSAO, a polyamine oxidase, in order to establish whether it is inactivated by the catalytically produced H_2O_2 as are the diamine oxidases from pea seedling (11) and pig kidney (12). A positive answer may be taken to imply that this type of inactivation is a general feature of amine oxidases, which is possibly part of an autoregolatory mechanism.

MATERIALS AND METHODS

BSAO was purified by the method of Wang *et al.* (13). The purified protein moved as a single band on SDS–PAGE. The concentration was measured spectrophotometrically at 280 nm, using $E = 1.74$ L g^{-1} cm^{-1} (14) and $M_r = 170$ kDa. The benzylamine oxidase activity

Abbreviations used: BSAO, bovine serum amine oxidase; SOD superoxide dismutase; TPQ, topaquinone, 2,4,5-trihydroxyphenylalanine quinone.

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was assayed at 25°C by monitoring the formation of benzaldehyde at 250 nm (ϵ = 12,500 M^{-1} cm^{-1}) (15). All samples had a minimum specific activity of 0.3 IU/mg (micromoles of substrate oxidized/min), which was lost upon titration with 1.00 ± 0.05 mol phenylhydrazine/dimer (ϵ = 41,500 M^{-1} cm^{-1} at 447 nm) (16). The copper content, assayed by atomic absorption spectrometry with a Perkin Elmer apparatus equipped with a HGA-400 graphite furnace, was of 2.0 ± 0.1 ions/dimer. The concentration of H_2O_2 in solution was measured by the absorbance (ϵ_{515nm} = 2.6 × 10^4 M^{-1} cm^{-1}) of the pink adduct produced by the horseradish peroxidase catalyzed oxidation of aminoantipirine followed by condensation with 3,5-dichloro-2-hydroxybenzensulfonic acid (17). Inactivation experiments were carried out by incubating BSAO with substrate in 0.1 M potassium phosphate, pH 7.2, at 37°C, in a thermostatted water bath, without shaking or stirring. Aliquots of the solutions, diluted to approximately 0.06 μM BSAO, were tested for activity with 5 mM benzylamine. For EPR experiments, 130 μM BSAO was inactivated by adding spermine, to a final concentration of 5.0 mM, immediately before it was transferred to the EPR tube, which was sealed with wax. BSAO was 85% inactivated in about 20 min, with formation of ≥0.23 mM H_2O_2.

Optical spectra were recorded with a Lambda-9 Perkin Elmer spectrophotometer. EPR spectra were recorded with a Brucker ESP300 spectrometer, operating in X band with a TM_{110}-type cavity. The sample was inserted in a 3 mm I.D. Suprasil tube (Wilmad), 300 μL volume, for the measurements at 100 K, and a Suprasil capillary tube (Wilmad), 50 μL volume, for the measurements at room temperature. EPR spectra were recorded at 1 mW and 10 mW microwave power, at 0.1 mT and 1 mT modulation amplitude, 3 ms/mT sweep time to sweep field ratio, 0.6 ms time constant. A Mn^{2+} standard sample, fixed at the bottom of the cavity, was used for field reference. A HP 53150A frequency counter was used for frequency measurements.

RESULTS

BSAO inactivation. BSAO at 0.6–130 μM was inactivated in air by all tested substrates at concentrations of 0.5–5.0 mM. Figure 1 reports an experiment in which 1 mL of 0.6 μM BSAO was incubated in a test tube open to air with four different substrates, and the residual activity measured at fixed time intervals. The loss of catalytic activity increased with time and with the substrate concentration. It was greater with spermine which has two reactive amino groups and smaller with phenylethylamine which has a lowest k_{cat} (18). It was pH-dependent, being larger at alkaline pH (Fig. 2). The presence of oxygen was required for inactivation, which did not occur when the substrate was added under anaerobic conditions. However, vigorous stirring, facilitating the oxygen diffusion in solution, protected the protein from inactivation even at high substrate concentrations. Like diamine oxidases (11, 12), BSAO was fully protected by catalase and was not inactivated by exogenous H_2O_2 in absence of substrate, either in the conditions of Table 1 (3 h incubation with 0.5 mM H_2O_2) or at longer time (24 h incubation with 0.5 mM H_2O_2). However, exogenous H_2O_2 added together with substrate contributed to the inactivation. Benzaldehyde addition up to 2 mM did not affect these reactions.

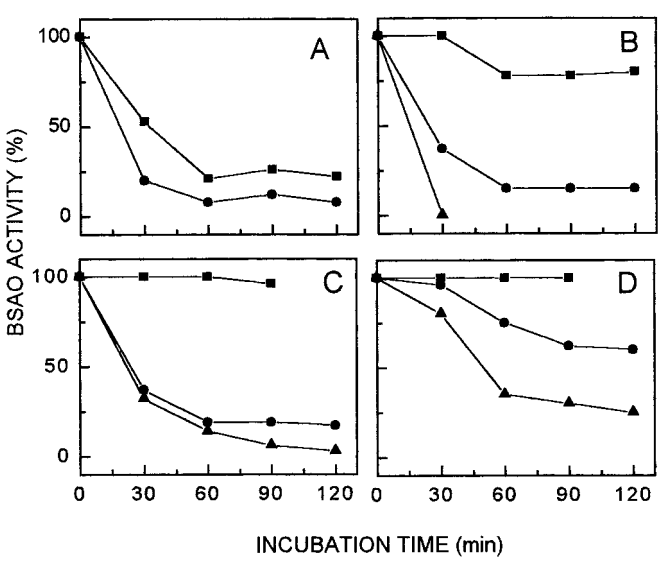

FIG. 1. Time course of BSAO inactivation by amines. 0.6 μM BSAO, in 0.1 M potassium phosphate buffer, pH 7.2, was incubated at 37°C with spermine (A), spermidine (B), benzylamine (C), and phenylethylamine (D). The substrate concentrations were 0.5 mM (■), 1.0 mM (●), 2.0 mM (▲).

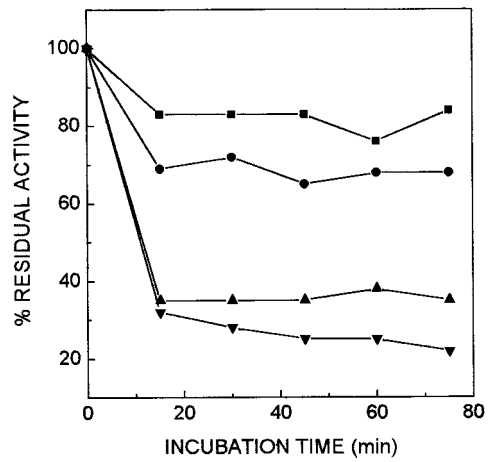

FIG. 2. Dependence on pH of BSAO inactivation. 0.6 μM BSAO was incubated at 37°C with 0.3 mM spermine in 0.1 M potassium phosphate buffer, pH 6.6 (■), 6.8 (●), 7.2 (▲), and 7.6 (▼).

TABLE 1

BSAO Inactivation by Spermine and H_2O_2

Spermine (mM)	Added H_2O_2 (mM)	Detected H_2O_2 (mM)	% Residual activity
1.0 + catalase	—	0.0	100
—	0.5	0.49	100
0.5	—	0.85	75
0.5	0.5	1.34	58
1.0	—	1.32	25

Note. The solutions were incubated for 1 h in air at 37°C in 0.1 M potassium phosphate buffer, pH 7.2.

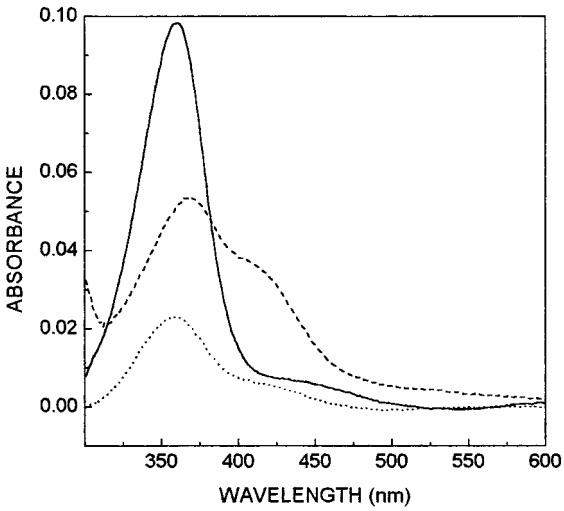

FIG. 3. Differential UV–Vis spectra of semicarbazide-reacted minus unreacted BSAO. 6 μM native BSAO, in 0.1 M potassium phosphate buffer, pH 7.2, was incubated 15 min with 0.5 mM semicarbazide (solid line); 6 μM BSAO, 85% inactivated by 20 min incubation with 2.0 mM spermine and dialyzed to remove oxidation products and excess spermine, was further incubated 15 min (dotted line) and 24 h (dashed line) with 0.5 mM semicarbazide.

The absorbance of the TPQ band at 480 nm decreased upon inactivation, while an increase of absorbance occurred below 400 nm, with a maximum around 310 nm in the differential spectrum (not shown). The latter band survived a 24-h dialysis, but disappeared after proteolytic digestion. The TPQ ability to bind reagents of the carbonyl group, such as phenylhydrazine or semicarbazide, decreased in the inactivated protein. In the case of semicarbazide, only the band at 360 nm formed by the first molecule reacting per BSAO dimer (Fig. 3, full line) was decreased (Fig. 3, dotted line). The slow reaction of ≈0.2 molecule per dimer (19) was almost unaffected (Fig. 3, dashed line).

Attempts to detect modified amino acids. In superoxide dismutases (SODs), the loss of catalytic activity, occurring on reaction with H_2O_2 produced in the catalytic cycle, was associated with the destruction of at least one conserved aminoacid in proximity of the active site (20, 21, and references therein). In Cu,Zn-SOD from bovine erythrocytes, amino acid analyses revealed the loss of one histidine per subunit, while a substantial change of the EPR spectrum allowed its identification as a Cu^{2+}-bound histidine (22). In Fe-SOD from *Porphyromonas gingivalis* a tryptophan and a cystein residue were lost in amino acid analyses (23). The tryptophan was identified as a conserved residue close to the active site by digesting the protein with lisylendopeptidase and sequencing the peptide that disappeared from the 280 nm HPLC elution profile upon inactivation (21). In the case of BSAO, only the EPR spectra provided some information, since attempts to identify modified residues by aminoacid analyses were frustrated by the protein large M_r, which made the experimental error of comparable magnitude as the eventual change produced by the destruction of an amino acid residue per dimer. It should be recalled that a single BSAO subunit is active (18, 19). Spectroscopic analyses showed no changes upon inactivation of the tryptophan content, this residues being not conserved in amine oxidases.

The EPR spectrum of native BSAO (A) and the spectra of the inactivated enzyme (B) recorded 15 min and 3 h after spermine addition, as described under Materials and Methods, are shown in Fig. 4. The two latter spectra were identical to each other and only slightly different from the spectrum recorded for native BSAO. Any intensity variation following inactivation was within the limits of experimental reproducibility (10%), after normalization for the dilution caused by substrate addition. The changes can be seen in the $g_\parallel$ region, where $A_\parallel$ is decreased by about 1 mT, and near 336 mT. The latter change is clearly due to the formation of a radical at $g = 2.001$ as it is more evident in the signal detected at 1 mW microwave power (Fig. 5B). The radical intensity, not exceeding a few percent that of the Cu^{2+} signal, was not appreciably enhanced at 298 K (Fig. 5A) and did not show any clear hyperfine structure.

DISCUSSION

The results of this study show for the first time that BSAO, a polyamine oxidase, is inactivated by substrate in the same way as the diamine oxidases reported previously (11, 12). The lack of inactivation when the substrate was added in anaerobic conditions or in the presence of catalase implies that the inactivating agent is H_2O_2 produced by the catalytic reaction. Exogenous H_2O_2 did not inactivate fully oxidized BSAO, either in absence of an amine substrate or under steady state

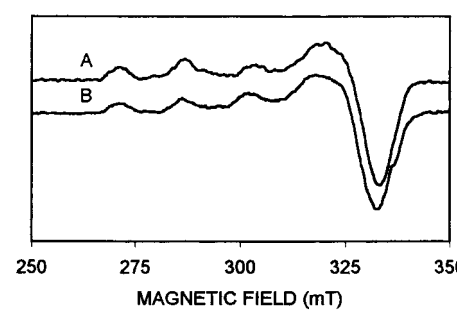

FIG. 4. EPR spectra of native and spermine-inactivated BSAO. 130 μM BSAO in 0.1 M potassium phosphate buffer, pH 7.2 (A); spermine added up to 5 mM concentration, spectrum recorded 15 min or 3 h after addition in air and corrected for dilution (B). Acquisition parameters: X-band, 10 mW microwave power, 1 mT modulation amplitude, 0.6 ms time constant, 3 ms/mT sweep time, 100 K temperature.

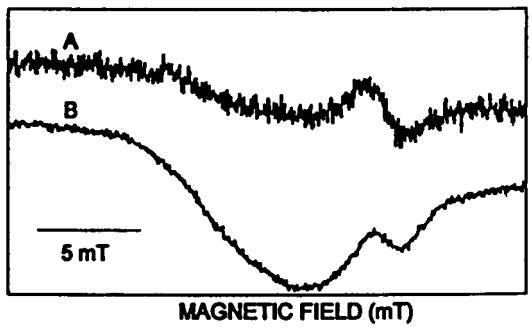

FIG. 5. EPR spectra of spermine-inactivated BSAO. The same solution of Fig. 5B was used. The spectra were recorded at 298 K and 10 mW microwave power (A); 100 K and 1 mW microwave power (B). Instrument conditions: X-band, 1 mW microwave power, 0.1 mT modulation amplitude. Other parameters as in Fig. 4.

conditions when oxygen exhaustion was prevented by stirring the solution in air. In such conditions BSAO is fully oxidized (A. Bellelli *et al.*, unpublished results), and H_2O_2 formed in the reoxidation step of the ping-pong reaction either is removed from the active site before the protein is again reduced or reacts at slower rate than oxygen. An influence of aldehydes on the reaction was not observed, in agreement with the full protecting effect of catalase and the inactivation by all tested substrates.

The irreversible reduction of TPQ on inactivation is strongly implied by (i) the formation in the UV–Vis spectrum of a band at 310 nm typical of reduced TPQ (24), which only disappeared upon peptidization; (ii) the small changes of the Cu^{2+} EPR spectrum, comparable with those induced by the reaction of TPQ with a pseudosubstrate (25); (iii) the loss of the ability to bind phenylhydrazine or semicarbazide (Fig. 4). Only the slow BSAO reaction with semicarbazide (19) was preserved (Fig. 3), confirming that this involves an inactive subunit. A loss of Cu^{2+} as a cause of stabilization of reduced TPQ (26), is ruled out by EPR spectra.

In Cu,Zn-SOD and Fe-SOD, the inactivation was proposed to proceed as a Fenton-like reaction. The protein metal ion is reduced by H_2O_2 with formation of superoxide, then this powerful oxidant attacks a nearby residue (27). Such a mechanism seems unlikely to occur in BSAO, unless it involves traces of spurious copper, since the protein Cu^{2+} is quite resistant to reduction, in particular by substrate (28). However, the similar behaviour of the three tested amine oxidases (11, 12) suggests that the H_2O_2 target is a conserved amino acid residue as it is in SODs. This residue, essential to the catalytic reaction, is sensitive to H_2O_2 only under reducing conditions and thus must be modified in some way by the reduction of TPQ. Four histidines in proximity of the active site are conserved (29); three are Cu^{2+} ligands and the fourth one is provided by the end of an hairpin arm with origin near the active site of the other subunit (30). However, the changes produced in the BSAO EPR spectrum by inactivation are much smaller than in the Cu,Zn-SOD spectrum, making the oxidation of any of the histidines unlikely, even by considering that the Cu^{2+} site of BSAO is probably less sensitive to modifications of the ligands than the strongly rhombic site of Cu,Zn-SOD (22). Another conserved residue at BSAO active site as possible H_2O_2 target is Tyr371, corresponding to Tyr369 in *E. coli* amine oxidase (29, 31) or Tyr305 in *Hansenula polimorpha* amine oxidase (32). This tyrosine is within hydrogen-bonding distance of the TPQ ionized C-4 hydroxyl and is likely to acquire a negative charge on reduction of TPQ, because a proton shift within the hydrogen bond may be induced by a considerable increase of the hydroxyl pK_a (33, 34). The modification of the tyrosine could make the shift irreversible, stabilizing the TPQ reduced form. The mutation of Tyr305 to Phe was recently shown to have a large inactivating effect on *Hansenula polimorpha* amine oxidase because of the modified hydrogen bond pattern around the active site (35). The present mechanism is quite different from that proposed for the inactivation of a yeast metylamine oxidase mutant, which was reversible and strictly substrate dependent (36).

The radical of low intensity found in the solutions of spermine inactivated BSAO is similar to the radical observed by Dooley *et al.* (37) and by Su and Klinman (24) in solutions of native BSAO that had been reacted with benzylamine under anaerobic conditions. At difference from the radical of Dooley *et al.* (37), the intensity of the present radical is not temperature-dependent. The assignment as a Cu^{+}–aminosemiquinone intermediate in equilibrium with Cu^{2+}–aminoquinol (24, 37) seems thus unlikely. It seems instead possible that the radical is an intermediate of the inactivation reaction since the the loss of catalytic activity (Fig. 2) and the radical intensity (24) show a similar pH dependence and since the radical persists in largely inactivated BSAO solutions. It cannot be excluded that the radical is due to an impurity or to a small amount of denatured molecules as a similar EPR signal was previously detected in solutions of half-Cu-depleted BSAO, which had been reduced with sodium dithionite, instead of substrate, in order to remove the copper with *N*,*N*-diethyldithiocarbamate (38).

Conclusions. Amine oxidases are ubiquitous enzymes involved in an increasing number of different functions. The same applies to H_2O_2 as a signal molecule or a crucial substrate. However, H_2O_2 can also induce, together with aldehydes, cytotoxic effects on the cells where it is produced (39). Thus, the inactivation reaction may be part of an *in vivo* process which modulates the amine oxidase activity and thereby the concentration of H_2O_2. The reaction may help explain some biochemical processes *in vivo*, for instance, that of cell adhesion in which a relationship was not found

between the VAP-1 protein adhesion and its very low or absent amine oxidase activity (9).

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REFERENCES

1. McIntire, W. S., and Hartmann, C. (1992) *in* Principles and Applications of Quinoproteins (Davidson, V. L., Ed.), pp. 97–171, Decker, New York.
2. Janes, S. M., Mu, D., Wemmer, D., Smith, A. J., Kaur, S., Maltby, D., Burlingame, A. L., and Klinman, J. P. (1990) *Science* **248,** 981–987.
3. Cai, D., and Klinman, J. P. (1994) *J. Biol. Chem.* **269,** 32039–32042.
4. Matsuzaki, R., Suzuki, S., Yamaguchi, K., Fukui, T., and Tanizawa, K. (1995) *Biochemistry* **34,** 4524–4530.
5. Wang, X., Pietrangeli, P., Mateescu, M. A., and Mondovì, B. (1996) *Biochem. Biophys. Res. Commun.* **223,** 91–97.
6. Langford, S. D., Trent, M. B., Balakumaran, A., and Boor, P. J. (1999) *Toxicol. Appl. Pharmacol.* **155,** 237–244.
7. Novotny, W. F., Chassande, O., Backer, M., Lazdunski, M., and Barbry, P. (1994) *J. Biol. Chem.* **269,** 9921–9925.
8. Mu, D., Medzihradszky, K. F., Adams, G. W., Mayer, P., Hines, W. M., Burlingame, A. L., Smith, A. J., Cai, D., and Klinman, J. P. (1994) *J. Biol. Chem.* **269,** 9926–9932.
9. Smith, D. J., Salmi, M., Bone, P., Hellman, J., Leu, T., and Jalkanen, S. (1998) *J. Exp. Med.* **188,** 17–27.
10. Rhee, S. G. (1999) *Exp. Mol. Med.* **31,** 53–59.
11. Mann, P. J. G. (1955) *Biochem. J.* **59,** 609–620.
12. Mondovì, B., Rotilio, G., Finazzi-Agrò, A., and Costa, M. T. (1967) *Biochim. Biophys. Acta* **132,** 521–523.
13. Wang, X. T., Dumulin, M. J., Befani, O., Mondovì, B., and Mateescu, M. A. (1994) *Prep. Biochem.* **24,** 237–250.
14. Suzuki, S., Sakurai, T., Nakahara, A., Manabe, T., and Okuyama, T. (1983) *Biochemistry* **22,** 1630–1635.
15. Tabor, C. W., Tabor, H., and Rosenthal, S. M. (1954) *J. Biol. Chem.* **208,** 645–661.
16. Morpurgo, L., Agostinelli, E., Mondovì, B., Avigliano, L., Silvestri, R., Stefancich, G., and Artico, M. (1992) *Biochemistry* **31,** 2616–2621.
17. Angelini, R., Rea, G., Federico, R., and D'Ovidio, R. (1996) *Plant Science* **119,** 103–113.
18. Agostinelli, E., De Matteis, G., Mondovì, B., and Morpurgo, L. (1998) *Biochem. J.* **330,** 383–387.
19. De Biase, D., Agostinelli, E., De Matteis, G., Mondovì, B., and Morpurgo, L. (1996) *Eur. J. Biochem.* **273,** 93–99.
20. Beyer, W. F., and Fridovich, I. (1987) *Biochemistry* **26,** 1251–1257.
21. Yamakura, F., Rardin, R. L., Petsko, G. A., Ringe, D., Hiraoka, B. Y., Nakayama, K., Fujimura, T., Taka, H., and Murajama, K. (1998) *Eur. J. Biochem.* **253,** 49–56.
22. Bray, R. C., Cockle, S. A., Fielden, E. M., Roberts, P. B., and Rotilio, G. (1974) *Biochem. J.* **139,** 43–48.
23. Yamakura, F. (1984) *Biochem. Biophys. Res. Commun.* **122,** 635–641.
24. Su, Q., and Klinman, J. P. (1998) *Biochemistry* **37,** 12513–12525.
25. Morpurgo, L., Agostinelli, E., Muccigrosso, J., Martini, F., Mondovì, B., and Avigliano, L. (1989) *Biochem. J.* **260,** 19–25.
26. Agostinelli, E., Morpurgo, L., Wang, C., Giartosio, A., and Mondovì, B. (1994) *Eur. J. Biochem.* **222,** 727–732.
27. Hodgson, E. K., and Fridovich, I. (1975) *Biochemistry* **14,** 5295–5299.
28. Mondovì, B., Finazzi-Agrò, A., Rotilio, G., and Sabatini, S. (1986) *in* Frontiers in Bioinorganic Chemistry (Xavier, A. V., Ed.), pp. 604–611, VCH, Weinheim, FRG.
29. Tipping, A. J., and McPherson, M. J. (1995) *J. Biol. Chem.* **270,** 16939–16946.
30. Parsons, M. R., Convery, M. A., Wilmot, C. M., Yadav, K. D. S., Blakeley, V., Corner, A. S., Phillips, S. E. V., McPherson, M. J., and Knowles, P. F. (1995) *Structure* **3,** 1171–1184.
31. Wilmot, C. M., Murray, J. M., Alton, G., Parsons, M. R., Convery, M. A., Blakeley, V., Corner, A. S., Palcic, M. M., Knowles, P. F., McPherson, M. J., and Phillips, S. E. V. (1997) *Biochemistry* **36,** 1608–1620.
32. Li, R., Klinman, J. P., and Mathews, F. S. (1998) *Structure* **6,** 293–307.
33. Bossa, M., Morpurgo, G. O., and Morpurgo, L. (1994) *Biochemistry* **33,** 4425–4431.
34. De Matteis, G., Agostinelli, E., Mondovì, B., and Morpurgo, L. (1999) *JBIC* **4,** 348–353.
35. Hevel, J. M., Mills, F. A., and Klinman, J. P. (1999) *Biochemistry* **38,** 3683–3693.
36. Cai, D., Dove, J., Nakamura, N., Saunders-Loehr, J., and Klinman, J. P. (1997) *Biochemistry* **36,** 11472–11478.
37. Dooley, D. M., Mc Guirl, M. A., Brown, D. E., Turowsky, P. N., Mc Intire, W. S., and Knowles, P. F. (1991) *Nature* **349,** 262–264.
38. Morpurgo, L., Agostinelli, E., Befani, O., and Mondovì, B. (1987) *Biochem. J.* **248,** 865–870.
39. Averill-Bates, D., Agostinelli, E., Przybytkoski, E., Mateescu, M. A., and Mondovì, B. (1993) *Arch. Biochem. Biophys.* **300,** 75–79.