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Nuclear Magnetic Resonance Study of the Exchangeable Histidine Protons in Bovine and Wheat Germ Superoxide Dismutases†

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ABSTRACT: Nuclear magnetic resonance studies at 220 MHz of the exchangeable histidine NH protons in bovine erythrocyte superoxide dismutase (BESOD) [EC 1.15.1.1] and the two isoenzymes of wheat germ superoxide dismutase (WGSODI and WGSODII) have been carried out. NMR spectral similarities reveal substantial structural homology of WGSOD with BESOD. Comparison of the spectra of the apoproteins and copper-free, native, and reduced proteins suggests that zinc has a structural role in WGSOD similar to that previously reported for BESOD [Lippard, S. J., Burger, A. R., Ugurbil, K., Pantoliano, M. W., & Valentine, J. S. (1977) *Biochemistry 16*, 1136]. Four resonances are assigned to conserved histidine residues, three of which are coordinated to the zinc atom while the fourth is nonligating.

Superoxide dismutases are widely distributed in nature where, it has been proposed, they serve to protect aerobic organisms from the toxic effects of the superoxide radical (Fridovich, 1978). The enzyme present in the cytosol of eukaryotes contains one copper and one zinc atom in each of two identical subunits. X-ray diffraction studies of the enzyme isolated from bovine erythrocytes, BESOD,[1] shows the metal ions to be 6 Å apart and bridged by a common ligand, His-61 (Richardson et al., 1975). The coordination sphere about the copper is a distorted square formed by the nitrogen atoms of the imidazole groups of His-44, His-46, His-118, and His-61, while the zinc is surrounded by a distorted tetrahedron of ligands donated by His-69, His-78, Asp-81, and His-61. In addition, the bovine erythrocyte enzyme contains two histidines which are not part of the active site, His-19 and His-41 (Steinman et al., 1974).

At present, no crystal structure has been determined for any other eukaryotic Cu–Zn superoxide dismutase. The entire amino acid sequence is apparently available for the enzyme isolated from baker's yeast, *Saccharomyces cerevisiae* (Cass et al., 1978). All seven of the metal binding amino acid residues are conserved whereas neither of the other two histidines, His-19 and His-41, is conserved. In the human erythrocyte enzyme, 67% of which has been sequenced, histidines-41, -46, -61, -69, and -118 are known to be conserved (Barra et al., 1978).

Wheat germ contains two isoenzymes of the copper zinc superoxide dismutase (Beauchamp & Fridovich, 1973). The isoenzymes differ from BSOD in amino acid composition (Beauchamp & Fridovich, 1973). There are likely to be other differences between BSOD and WGSOD since it has been reported that plant and mammalian copper zinc superoxide dismutases are immunologically distinct (Asada et al., 1976).

Previous NMR studies on BESOD (Lippard et al., 1977a; Cass et al., 1977) and BLSOD (Stoesz et al., 1979) support the idea that the solution structure is the same as that in the solid as determined by X-ray crystallography and that NMR spectroscopy is an excellent probe of metal binding at the active site (Cass et al., 1979a; Stoez et al., 1979). The NMR spectrum of yeast superoxide dismutase has also been studied, and the results were used to show the similarities between the active-site structures of the bovine and yeast enzymes (Cass et al., 1978). The C2–H proton resonances of the copper and zinc binding histidines in BSOD and yeast superoxide dismutase have been assigned (Cass et al., 1979b).

Here we report the NMR spectra of WGSOD and BESOD in the region of the exchangeable histidine NH protons as a function of pH and metal binding. The results reveal considerable active-site structural homology between the bovine and wheat germ enzymes. In addition, assignments are made for several of the exchangeable NH resonances in the three proteins studied.

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[1] Abbreviations used: BESOD, bovine erythrocyte superoxide dismutase; BLSOD, bovine liver superoxide dismutase; BSOD, bovine superoxide dismutase; WGSODI, wheat germ superoxide dismutase, isoenzyme I; WGSODII, wheat germ superoxide dismutase, isoenzyme II; NMR nuclear magnetic resonance; NOE, nuclear Overhauser effect.

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Materials and Methods

BESOD was isolated and prepared for NMR spectroscopy as described previously (Lippard et al., 1977a). Wheat germ was obtained from Sigma Chemical Co., grade III. WGSOD was prepared by a modification of the method of Beauchamp & Fridovich (1973). The active fractions I and II obtained from the DEAE column were dialyzed against deionized water and lyophilized. The lyophilized powders were dissolved in ~10 mL of 1 mM potassium phosphate, pH 7.1, buffer and passed through a 2.5 × 30 cm Sephadex G-75 column equilibrated with the same buffer. Active fractions were dialyzed against deionized water and lyophilized.

Disc gel electrophoresis, performed according to the method of Ornstein (1964), showed WGSODII to be homogeneous and WGSODI to be contaminated with a small amount of WGSODII. The ESR spectra of WGSODI and WGSODII were very similar (Burger, 1979) and closely matched those reported in the literature for BESOD (Keele et al., 1971), with the exception that a small signal near $g = 4$ was detected (see below). Atomic absorption photometry showed both WGSODI and WGSODII to contain 0.8–0.9 equiv of copper and zinc per subunit as well as 0.1–0.2 equiv of iron (see below). Superoxide dismutase activity was measured by the hydroxydopamine assay (Heikkila & Cabbat, 1976). Both WGSODI and WGSODII had specific activities similar to that of BESOD.

The wheat germ proteins, in our hands, had a brownish green color not reported previously. We attribute the brown component to iron bound as an impurity, as revealed by atomic absorption and the $g \sim 4$ signal in the ESR spectrum. The molecular weights and amino acid compositions (Burger, 1979) of the WGSOD used in the present investigation are essentially the same as those reported previously (Beauchamp & Fridovich, 1973).

Apoprotein and zinc-free and dithionite-reduced proteins were prepared as described previously (Lippard et al., 1977a; Valentine et al., 1979). Copper-free proteins were prepared from the native proteins (Rotilio et al., 1977) or by addition of zinc sulfate to the apoproteins. Atomic absorption spectroscopy showed that the apoproteins contained less than 0.05 equiv of copper or zinc per subunit. Zinc-free BESOD contained less than 0.05 equiv of zinc and 0.9 equiv or more of copper per subunit. The copper-free proteins retained no less than 90% of the zinc they originally contained and had less than 5% residual copper.

NMR spectra were obtained on a Varian HR-220 spectrometer operating in the continuous wave mode, as described previously (Lippard et al., 1977a). The pH was adjusted either with 1 N NaOH or 1 N HCl. Atomic absorption measurements were made on a Varian AA-365 or a Techtron AA4 instrument. All spectrophotometric measurements were performed on a Cary 118 C spectrophotometer. 6-Hydroxydopamine was a product of Regis Chemical Co., Chicago. Doubly distilled, deionized water and reagent-grade chemicals were used throughout.

Results

The proton NMR spectra of several forms of BESOD are shown in Figure 1. As reported previously, these peaks are assigned to histidine NH protons in slow exchange with solvent (Lippard et al., 1977a; Stoesz et al., 1979). Figure 2 compares the spectra of dithionite reduced with the copper-free WGSODI enzyme and demonstrates that the protein may be reconstituted by addition of cupric ion to the copper-free enzyme. A similar study with WGSODII is shown in Figure 3. The pH dependence of the chemical shifts of the resonances in reduced BESOD observed by us (Burger, 1979) is virtually identical with that reported previously (Stoesz et al., 1979).

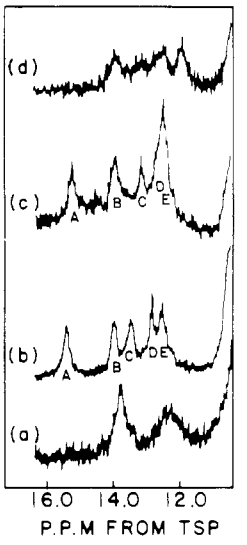

FIGURE 1: 220-MHz proton NMR spectra of BESOD in 50 mM potassium phosphate buffer. (a) Oxidized, pH 6.0; (b) reduced, pH 6.0; (c) copper free, pH 6.0; (d) apo, pH 6.0. For details see Lippard et al. (1977a).

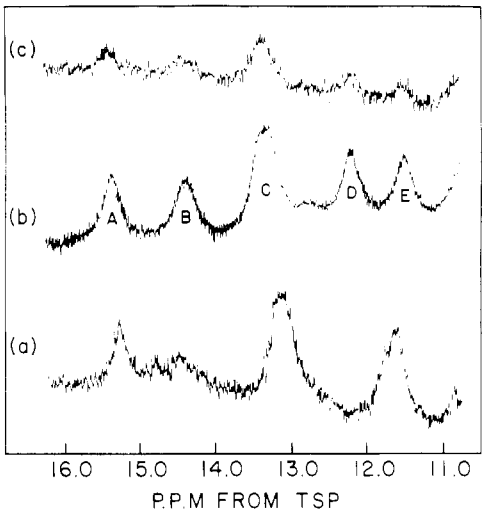

FIGURE 2: NMR spectra of WGSODI in 100 mM potassium phosphate buffer. (a) Copper free, pH 7.3; (b) reduced, pH 6.4; (c) after addition of 1.0 equiv of copper(II) acetate per subunit to the copper free protein, followed by reduction with dithionite, pH 6.7.

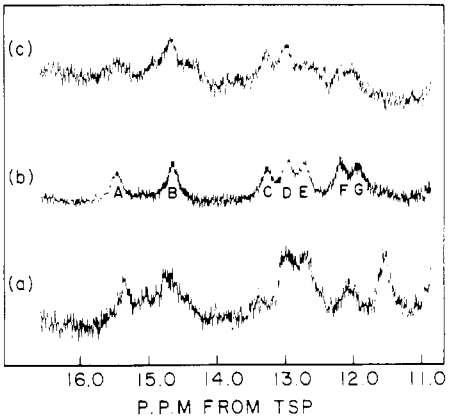

FIGURE 3: NMR spectra of WGSODII in 100 mM phosphate buffer. (a) Copper free, pH 7.2; (b) reduced, pH 5.8; (c) reconstituted from copper-free protein and reduced as in Figure 2c, pH 5.8.

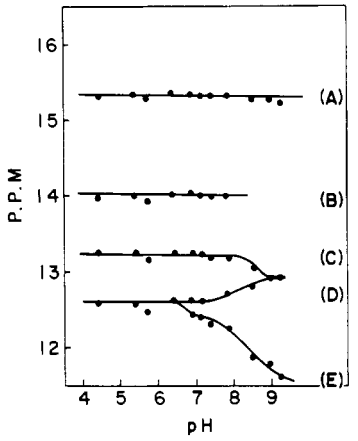

FIGURE 4: pH dependence of the chemical shifts in copper-free BESOD in 50 mM potassium phosphate buffer. The pH variation over the time required to accumulate the spectra was less than 0.1 pH unit in all the spectra. The lines through the data points were hand drawn. Resonances are labeled as shown in Figure 1c.

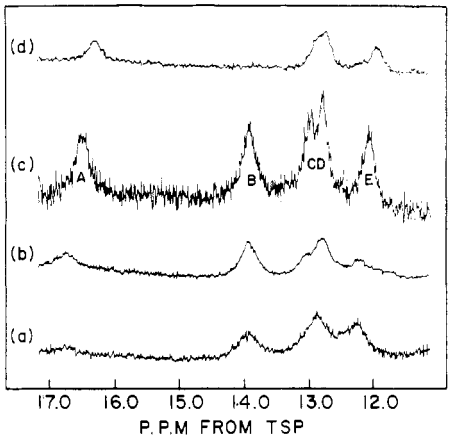

FIGURE 5: NMR spectra of reduced zinc-free BESOD in 50 mM potassium phosphate buffer at (a) pH 4.8, (b) pH 5.2, (c) pH 7.2, and (d) pH 9.2.

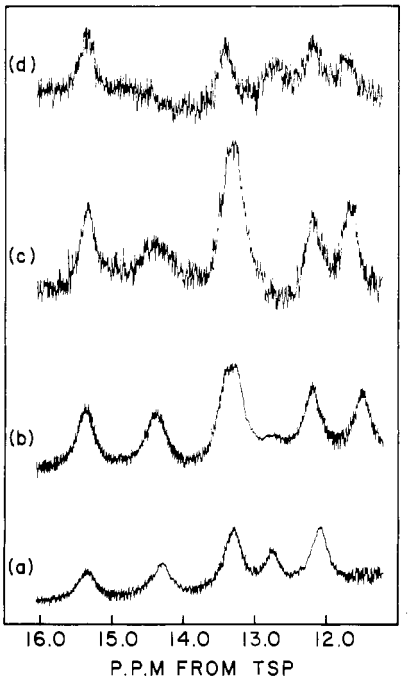

FIGURE 6: NMR spectra of reduced WGSODI in unbuffered solutions at (a) pH 4.9, (b) pH 6.4, (c) pH 7.5, and (d) pH 9.8.

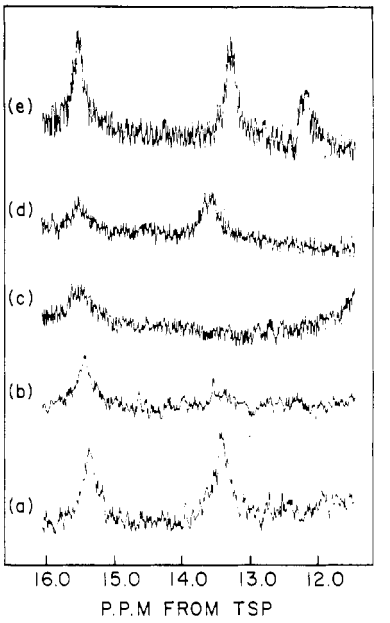

FIGURE 7: NMR spectra in D_2O (pH values are uncorrected for the deuterium isotope effect) of (a) reduced BESOD, pH 5.7, after standing for 2 h at room temperature, (b) reduced BESOD as in (a) but with subsequent heating for 2.5 h at 45 °C, (c) copper-free BESOD, pH 5.4, after standing for 2 h at room temperature, (d) WGSODI, reduced, pH 5.7, after standing for 1.5 h at room temperature, and (e) reduced WGSODII, pH 5.9, after standing for 2 h at room temperature.

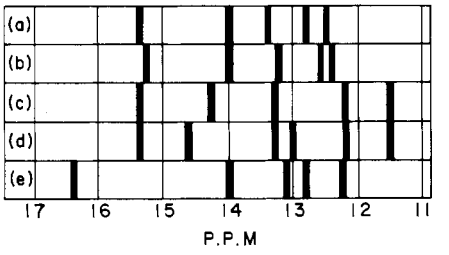

FIGURE 8: Summary of proton NMR chemical shifts for (a) reduced BESOD, pH 6.0, (b) copper-free BESOD, pH 7.0, (c) reduced WGSODI, pH 6.2, (d) reduced WGSODII, pH 7.6, and (e) reduced zinc-free BESOD, pH 6.2.

Analogous results for copper-free BESOD are shown in Figure 4. The NMR spectra of reduced, zinc-free BESOD at several pH values are shown in Figure 5. Figure 6 displays the NMR spectra of reduced WGSODI at several pH values. Similar results were observed for reduced WGSODII (Burger, 1979). The spectra of BESOD, WGSODI, and WGSODII in 99.8% D_2O are shown in Figure 7. The peaks seen in Figure 7 represent those resonances with very long deuterium exchange times (several hours) under the conditions described in the figure caption. A diagram summarizing the chemical shifts of various resonances in BESOD, WGSODI, and WGSODII is presented as Figure 8.

Discussion

The apo forms of BESOD, WGSODI, and WGSODII show either broad, poorly resolved resonances or no detectable peaks. This result has been attributed to rapid exchange of the histidine NH protons with solvent (Lippard et al., 1977a). The presence of zinc alone leads to a well-resolved spectrum similar to that of the reduced enzymes (compare parts b and c of Figure 1; parts a and b of Figure 2, and parts a and b of Figure 3). These results indicate that the apoproteins have a flexible, solvent accessible structure which can be organized by zinc

alone into a conformation similar to that present in the reduced native enzyme. In the case of BESOD this conclusion has been supported by chemical modification (Lippard et al., 1977a) and histidine C2–H NMR spectral (Cass et al., 1979a) studies. The similarities in the NMR spectra of the reduced forms of all three proteins (Figures 1b, 2b, and 3b) suggest analogous active-site structures. Moreover, the results presented here indicate that zinc is instrumental in organizing the active-site structure in both WGSODI and WGSODII just as in BESOD (Figures 1–3).

Assignment of Resonances. The convention previously used (Stoesz et al., 1979) in naming the resonances of BLSOD is adopted here for both BESOD and WGSOD. Thus, the resonances in reduced BESOD are labeled, starting from the most downfield peak, as A, B, C, D, and E (Figure 1).

(1) Resonance B in BESOD, WGSODI, and WGSODII. This peak in BLSOD has been previously assigned (Stoesz et al., 1979) to the N3 proton of His-41 by means of NOE and deuterium exchange experiments. We concur in this assignment and present additional evidence to support it.

Resonance B in BESOD is always present irrespective of the metal content (Figures 1 and 5). Since its chemical shift is unaffected by the presence of metals, it appears to be due to a histidine far from the active site and, hence, not to a ligand. In addition to peak B being present in all forms of BESOD examined, this resonance always exhibits the same behavior as a function of pH (Figures 4 and 5). The resonance disappears above pH 8, probably due to rapid, base-catalyzed exchange (Stoesz et al., 1979).

The spectra of both isoenzymes of the wheat germ proteins exhibit a peak close to, but not identical with, the position of peak B in the BESOD enzyme (Figure 8). Interestingly, this resonance has a pH dependence analogous to that of peak B in BESOD (Figure 6). Removal of copper has no effect on the chemical shift of peak B in both WGSODI and WGSODII (Figures 2 and 3).

The chemical shifts of peak B in the three proteins, BESOD, WGSODI, and WGSODII, although similar, are distinctive. We propose that peak B in WGSODI and WGSODII originates from a histidine far removed from the active site, i.e., similar to His-41 in BESOD. This residue therefore seems to be conserved in the three proteins, although its detailed microenvironment must differ somewhat in order to account for the slight chemical shift differences.

(2) Resonance A in BESOD, WGSODI, and WGSODII. This resonance in BESOD has been assigned to the N3 proton of the zinc ligand, His-69, based on a combination of pH titration, chemical exchange, and NOE experiments in conjunction with inspection of the crystal structure (Stoesz et al., 1979). Again, we agree with this assignment and present supporting evidence for it below.

Resonance A is only present in those BESOD derivatives that contain zinc and is characteristically absent when zinc is absent (Figures 5 and 8). In all cases where this peak is present it fails to undergo any changes as a function of pH [Figure 4 and Stoesz et al. (1979)]. Further confirmation of its assignment to a zinc ligand is the observation (Stoesz et al., 1979) that resonance A exhibits an NOE to a resonance at 7.71 ppm which has been assigned (Cass et al., 1979b) to a C2–H proton on a histidine coordinated to zinc.

Both WGSODI and WGSODII have a peak corresponding closely in chemical shift with resonance A in BESOD (Figure 8). This result is consistent with the previous assignment (Stoesz et al., 1979) of resonance A to His-69 in BSOD since, being a zinc ligand at the active site, it is expected to be conserved. The assignment of resonance A to a zinc ligand is further supported by the observation that this peak, in both WGSODI and WGSODII, fails to titrate (Figure 6) and exchanges only very slowly with D_2O (Figure 7). This behavior is the same as that of peak A in BESOD.

(3) Peak C in BESOD, WGSODI, and WGSODII. This resonance in BLSOD has been assigned to the N3 proton of the copper ligand His-44 by essentially the same arguments used to assign resonance A (Stoesz et al., 1979). However, as recognized by the authors (Stoesz et al., 1979), it is not possible based on the available data to rule out assignment of this peak (or peak A) to the zinc ligand His-78. Furthermore, resonance C shows an NOE (Stoesz et al., 1979) to a resonance at 7.7 ppm which was subsequently assigned (Cass et al., 1979b) to the C2–H proton of a histidine coordinated to zinc. While our data do not provide an unambiguous choice between these two possibilities, we tentatively favor assignment of peak C to a histidine coordinated to zinc because, like resonance A, it appears in the spectrum of the copper-free protein and is absent (or shifted) when zinc is absent (Figure 8).

The wheat germ proteins contain resonances very close in chemical shift to that of peak C in BESOD (Figure 8). In WGSODI there is at least one additional underlying resonance (Figure 2b) which is better resolved into two separate peaks in WGSODII (Figure 3b, peaks labeled D and E). Peak C in both WGSODI and WGSODII fails to titrate with pH and exchanges slowly with D_2O (Figures 6 and 7), consistent with an assignment to a conserved metal ligand. The crystal structure of BESOD (Richardson et al., 1975) shows the zinc to be buried, and it is expected to have limited solvent accessibility. This feature is consistent with the slow exchange with D_2O of the NH protons of histidine coordinated to zinc. In summary, the histidine residues giving rise to resonances A and C are conserved in the three proteins.

(4) Other Resonances. Peak E in reduced BLSOD was attributed to a metal coordinating histidine although no specific residue was assigned (Stoesz et al., 1979). Resonance E shows an NOE to a peak at 8.44 ppm which has been assigned (Cass et al., 1979b) to a C2–H proton from a histidine coordinated to zinc. Both reduced WGSODI and WGSODII have resonances at ~12.2 ppm, D and F, respectively, somewhat upfield of E in reduced BESOD (Figures 2, 3, and 8), which are similarly assigned to coordinated histidines since their chemical shifts exhibit no pH dependence (Figure 6). Both peak E in BESOD and the upfield nontitrating resonance D in WGSODI undergo rapid deuterium exchange compared to resonances A and C (Figure 7). In WGSODII peak F is still detectable in D_2O although its intensity is lower than those of resonances A and C.

A possible specific assignment for this resonance is the N–H proton of His-61, which bridges copper and zinc in the native oxidized protein but which might not bridge these metals in the reduced protein. In one proposed mechanism for BESOD (Hodgson & Fridovich, 1975; Lippard et al., 1977b), this residue becomes coordinated exclusively to zinc in the reduced form of the enzyme. Presumably, its environment would then be more accessible to solvent since it is closest to the exposed copper site. It is therefore reasonable that the NH proton from His-61 would exchange rapidly with D_2O in BESOD and WGSOD.

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Comparison of the β-Galactosidase Conformations Induced by D-Galactal and by Magnesium Ions†

O. M. Viratelle‡ and J. M. Yon*

ABSTRACT: The interaction of β-galactosidase with D-galactal, a "transition-state analogue", is strongly dependent upon the presence of Mg ions. In the presence of 1 mM Mg^{2+}, D-galactal interacts with β-galactosidase with a K_I value of 5 μM. The binding of D-galactal can be analyzed as a single exponential process occurring with a rate constant of 4.5×10^2 M^{-1} s^{-1}. This low value, along with spectral difference evidence, indicates the existence of an isomerization step, probably occurring after the formation of the Michaelis complex. The reappearance of free enzyme was tested by recording the appearance of *o*-nitrophenol during *o*-nitrophenyl β-galactoside hydrolysis. It occurs as a single exponential phenomenon ($k = 2.5 \times 10^{-3}$ s^{-1}) and can be accounted for mainly by the hydration of the enzyme–D-galactal complex. In the absence of magnesium, D-galactal exhibits an increase of the K_I value by 3 orders of magnitude. This effect arises from a large decrease in the binding rate, which precludes the study of binding kinetics. The hydration of the D-galactal–enzyme complex occurs in a time range similar to the one observed in the presence of Mg ions. But the reappearance of enzyme activity, as recorded by the appearance of *o*-nitrophenol, is no longer a simple process: following an activation process, a decrease of the enzymatic activity is observed. By quantitative analysis, it is shown that the enzyme exhibits temporarily a higher activity than it usually does in the absence of magnesium. The rate of the inactivation step is very similar to the one observed when Mg-activated enzyme isomerizes into Mg-free enzyme, suggesting that D-galactal induces an active conformation state similar to the one induced in the presence of Mg ions. These data are discussed in the light of the known positive influence of Mg ions upon an isomerization step during enzyme catalysis.

β-Galactosidase from *Escherichia coli* is characterized by a molecular weight unusually large for its protomer [116 349; Fowler & Zabin (1978)] and by a turnover number relatively high for a glycosidic enzyme [k_{cat} = 1300 s^{-1} for its best substrates, dinitrophenyl galactosides; Sinnott & Viratelle (1973)]. The relationship between these two characteristics is not known. Probably, this long polypeptide chain favors, either in a static or in a dynamic fashion, an optimal positioning of the residues of the active site. What is now assumed is that, during the catalytic hydrolysis of most of the aryl galactosides, a rate-limiting conformational change occurs (Viratelle & Yon, 1973; Sinnott & Souchard, 1973; Fink, 1977), which allows the chemical reaction to proceed with a faster rate via an acid-catalyzed departure of the aglycon (Sinnott et al., 1978; Sinnott, 1978).

Such a positive effect of a conformational change is observed only in the presence of a divalent cation, Mg^{2+} (probably other activator cations have the same effect). In its absence, however, the enzyme does retain some catalytic activity (Tenu et

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