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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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al., 1972; Withers et al., 1978). The study of the secondary isotopic effect and of the dependence of the catalytic constant upon the pK of the leaving group shows an indication that no conformational change occurs, and no acid catalysis can be evidenced (Sinnott et al., 1978). It has been demonstrated that the activating effect of magnesium is mediated through a change of the conformation of the enzyme, with a rate constant very low compared to the turnover of the enzyme. Moreover, this change can occur principally via the substrate-free enzyme; the formation of the Michaelis complex slows down or totally inhibits such a transition (Tenu et al., 1972).

In order to obtain more information about the chemical intermediate formed during the catalysis, Lee (1969) studied the inhibition of β-galactosidase by D-galactal. Now, more data concerning this interaction have been reported (Wallenfels & Weil, 1972; Lehmann & Schröter, 1972; Wentworth & Wolfenden 1974; Lehmann & Ziegler, 1977). It has been shown that D-galactal does not behave like other known substrates or inhibitors of the enzyme in the sense that its binding and release are slow phenomena. It is, however, a substrate of the enzyme, being hydrated to 2-deoxygalactose or forming 2-deoxygalactosyl derivatives by the addition of nucleophilic compounds.

The low rate constants are comparable to the ones described previously for the Mg^{2+}-induced conformational changes (Tenu et al., 1972). The kinetic work of Wentworth & Wolfenden (1974) was performed in the presence of 1 mM Mg^{2+} ions, which is a saturating concentration during the hydrolysis of aryl galactosides. To test a possible relationship between the effect of D-galactal and of Mg^{2+} on the enzyme, we carried out comparative studies of the interaction between D-galactal and β-galactosidase, in the presence and in the absence of Mg^{2+} ions. The results of these studies are presented here. A preliminary account has been reported previously (Viratelle et al., 1975).

We first fully characterized our system according to its equilibrium and to the kinetic parameters for the binding and release of D-galactal in the presence of 1 mM $MgSO_4$. Identical studies were then performed in the presence of EDTA. To gain more insight into the nature of the kinetic steps, we have also performed experiments at various substrate concentrations and in the presence of methanol. Methanol, which is a nucleophilic compound, acts as water does; therefore, its presence enhances the rate constants of any step in which water is implicated as a nucleophilic reactant (Viratelle & Yon, 1973). The substrate concentration influences the rate of Mg^{2+}-dependent conformational changes, since they operate mainly through the free enzyme. Our data show that D-galactal does not compete with Mg^{2+}. On the contrary, in the absence of Mg^{2+}, the interaction with D-galactal induces (or involves) an active state of the enzyme similar (if not identical) to the one obtained in the presence of Mg^{2+}. These results underline the role of Mg^{2+} in modulating the catalytic activity of β-galactosidase. This cation has been shown to favor the hydrolysis of aryl galactosides by promoting the occurrence of a conformational change during the catalysis (Sinnott et al., 1978) and to inhibit the hydrolysis of galactosylpyridinium salts (Sinnott et al., 1975; Withers et al., 1978). None of these effects is comparable, in magnitude, to the activating effect of Mg^{2+} on the interaction of D-galactal and β-galactosidase.

Materials and Methods

The β-galactosidase preparation and the experimental conditions used in the presence of 1 mM Mg^{2+} have been described previously (Tenu et al., 1971). Studies in the absence of Mg^{2+} were performed according to Tenu et al. (1972). D-Galactal was a product of EGA (distributed by Pierce Chemical Co.).

Statistical Treatment of Experimental Data. The interaction between D-galactal and β-galactosidase was mainly monitored by measuring the inhibition of hydrolysis of a chromogenic substrate (*o*-nitrophenyl galactoside or *o*-nitrophenyl fucoside). The absorbance change, corresponding to the appearance of *o*-nitrophenol, was recorded. From these data, a determination of the kinetic parameters was obtained after a preliminary estimation of their values by graphical methods, by using a statistical treatment according to Cleland (1967) adapted to a Wang 2200 calculator.

Two different analyses were used: (1) when the data could be accounted for by an exponential and a linear phenomena, the increase of the absorbance was described by

$$P = v_\infty t - (v_\infty - v_0)(1/k)(1 - e^{-kt}) \quad (1)$$

where v_0 is the velocity at zero time, v_∞ is the steady-state velocity at the end of the exponential phenomenon, and k is the exponential constant; (2) when the data suggested two exponential phenomena, the increase of the absorbance was described by

$$P = A(1 - e^{-k_A t}) + B(1 - e^{-k_B t}) + Ct \quad (2)$$

In this case, only k_A and k_B, the two exponential constants, were determined. In each case, a theoretical curve, calculated by using the determined parameters, was compared to the experimental curve to detect any significant discrepancy.

Determination of the Inhibition Constant for D-*Galactal.* The K_I constant for D-galactal was determined by taking into account the slow release of free enzyme from the D-galactal–enzyme complex. Enzyme and various D-galactal concentrations were incubated for 30 min, and a small aliquot of the mixture was introduced into the reaction solution in the presence of 1.33 mM *o*-Np-Gal.[1] The appearance of the product, *o*-nitrophenol, was recorded with a Cary-16 spectrophotometer until no more than 25% of the substrate was hydrolyzed. The reaction followed a slow exponential activation process; the experimental curve was analyzed according to eq 1, and v_0 and v_∞ were determined. Under these conditions, v_∞ is proportional to the total enzyme, and v_0 is proportional to the enzyme not involved in a complex with D-galactal at zero time. Therefore, the K_I constant for D-galactal is given by

$$K_I = \frac{[E_{free}][\text{D-galactal}]}{[E_{bound}]} = \frac{v_0[\text{D-galactal}]}{v_\infty - v_0} \quad (3)$$

Kinetic Study of the Binding of D-*Galactal.* Interaction of D-galactal and β-galactosidase was studied by introducing, at zero time, β-galactosidase into a solution containing varying concentrations of D-galactal and an *o*-nitrophenyl substrate (*o*-Np-Fuc or *o*-Np-Gal) whose concentration was equal to its Michaelis constant. The appearance of *o*-nitrophenol was recorded either with a Cary-16 (with *o*-Np-Fuc as the substrate) or with a Durrum stopped-flow apparatus (with *o*-Np-Gal as the substrate) coupled with a Biomation. The data were analyzed according to eq 1.

Release of D-*Galactal.* A preincubated mixture of β-galactosidase and D-galactal was introduced into an *o*-Np-Gal solution whose concentration was at least 10 times K_m, and the appearance of *o*-nitrophenol was followed with a Cary-16

[1] Abbreviations used: *o*-Np-Gal, *o*-nitrophenyl β-D-galactopyranoside; *o*-Np-Fuc, *o*-nitrophenyl β-D-fucopyranoside; EDTA, ethylenediaminetetraacetic acid; Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino]ethanesulfonic acid.

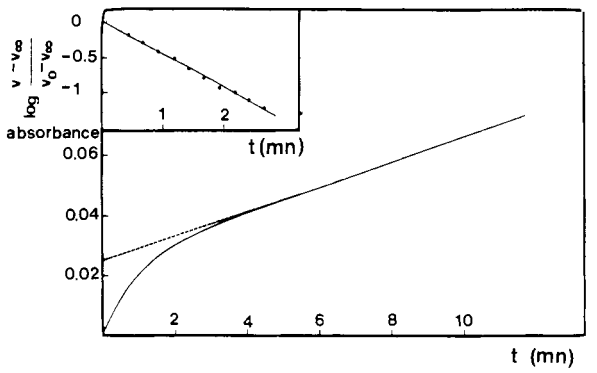


FIGURE 1: Effect of D-galactal on the enzymatic hydrolysis of an *o*-nitrophenyl substrate. Experimental conditions: β-galactosidase was introduced at zero time in a solution containing 3.1 mM *o*-Np-Fuc and 65 μM D-galactal, in 35 mM Tes, pH 7.0, 0.145 M NaCl, and 1 mM $MgSO_4$. The absorbance was recorded at 373 nm. The insert shows a semilogarithmic plot of the rate variation.

spectrophotometer. The data were analyzed according to eq 1 or 2.

Hydration of D-*Galactal.* Hydration of D-galactal in 2-deoxygalactose was followed by measuring the decrease of absorbance at 210 nm. At this wavelength, the molar extinction coefficient of D-galactal was found to be equal to 3250 M^{-1} cm^{-1}, and 2-deoxygalactose was transparent. Covered cells of 1-cm path length were used in a Cary-16 spectrophotometer. The experiments were carried out with 0.144 mM D-galactal in the presence of 145 mM NaCl, 1 mM $MgSO_4$, and 0.7 mM Tes, pH 7.

Results and Discussion

(I) Study of the Action of D-*Galactal in the Presence of 1 mM Mg^{2+} Ions. Equilibrium Constant for Interactions between* D-*Galactal and the Enzyme.* When β-galactosidase was preincubated with an excess of D-galactal and introduced into an *o*-Np-Gal solution, no initial hydrolysis of this substrate could be detected. Therefore, the initial hydrolysis rate can be used to determine the degree of inhibition of the enzyme after preincubation with nonsaturating concentrations of D-galactal. The binding constant for D-galactal was determined as described under Materials and Methods by using D-galactal concentrations from 1.5 to 25 μM. In all cases, the K_I value was close to 5 μM.

Kinetic Study of the Binding of D-*Galactal.* The rate constant for the interaction of D-galactal and β-galactosidase was determined by mixing the enzyme with D-galactal in the presence of a substrate, as described under Materials and Methods. Figure 1 shows a typical run; an exponential phenomenon occurs in addition to the stationary phase. The curves were analyzed according to eq 1. The exponential constant, *k*, was determined for various D-galactal concentrations (Figure 2a,b). No saturation occurred, at least until 7 mM. Since the substrate concentration was chosen equal to K_m, the bimolecular rate constant can be determined by multiplying the experimental slope by 2. The calculated k_{on} is 4.5×10^2 M^{-1} s^{-1}. This value is slightly modified by the presence of methanol; with 0.66 M methanol, the corresponding constant is 4.05×10^2 M^{-1} s^{-1}. It is also totally independent of magnesium concentration when the metal ion is varied from 0.05 to 3.3 mM in the enzymatic assay.

Kinetic Study of the Release of D-*Galactal.* When β-galactosidase, preincubated with D-galactal, was introduced into *o*-Np-Gal solution, a slow exponential phase was also observed (Figure 3). The corresponding constant, k_{off}, was determined as described under Materials and Methods and was found to

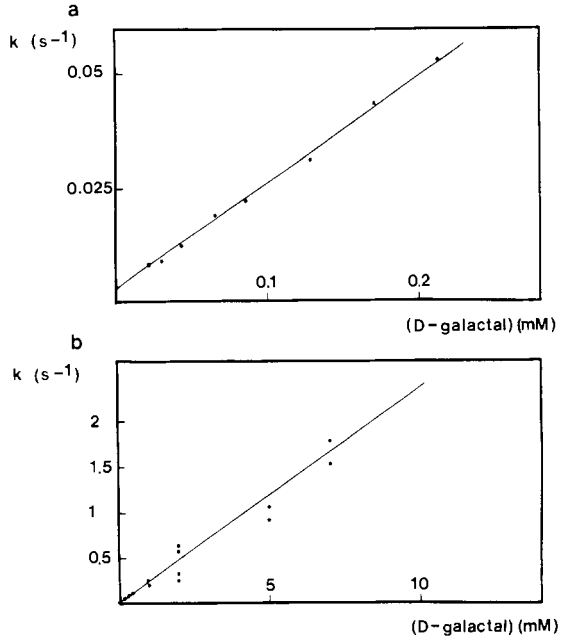


FIGURE 2: Effect of D-galactal concentration on the exponential constant of inactivation of β-galactosidase. Experimental conditions are as described in Figure 1 for (a). In (b), 0.1 mM *o*-Np-Gal was used instead of 3.1 mM *o*-Np-Fuc.

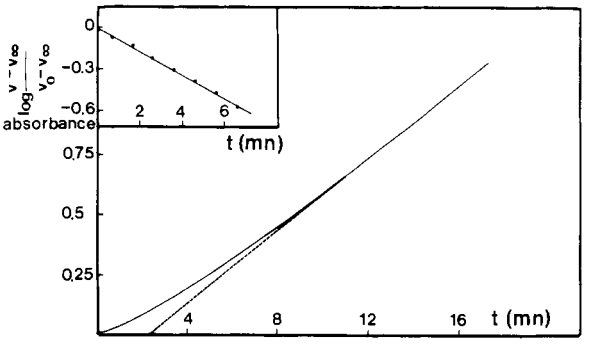


FIGURE 3: Enzymatic hydrolysis of *o*-Np-Gal after previous incubation of the enzyme with D-galactal. Experimental conditions: 5 μL of enzyme, incubated in the presence of 14 μM D-galactal, 10 mM Tes, pH 7.0, 0.145 M NaCl, and 1 mM $MgSO_4$, were introduced at zero time in 3 mL of 1.33 mM *o*-Np-Gal, 33 mM Tes, pH 7.0, 0.145 M NaCl, and 1 mM $MgSO_4$. The absorbance was recorded at 373 nm. The insert shows a semilogarithmic plot the rate variation.

Table I: Rate of Hydration of D-Galactal[a]

$[E_t]$ (μM)[b]	$k_{cat} \times 10^3$ (s^{-1})
0.13	2.2
0.23	2
0.26	2

[a] Hydration was studied as described under Materials and Methods. [b] As the protomer of M_r 116 350.

be equal to 2.5×10^{-3} s^{-1}. This constant is independent of substrate concentration and of Mg^{2+} concentration in the enzymatic assay. It increases linearly in the presence of methanol or mercaptoethanol (Figure 4).

Hydration of D-*Galactal.* Hydration of D-galactal was directly observed by a decrease in the absorbance at 210 nm. The k_{cat} values determined in three different experiments are reported in Table I. These values are in good agreement with the previous k_{off} value.

Discussion. In the presence of 1 mM Mg^{2+} ions, the effect of D-galactal on *o*-nitrophenyl galactoside hydrolysis is identical with that expected from this slow inhibitor as described by

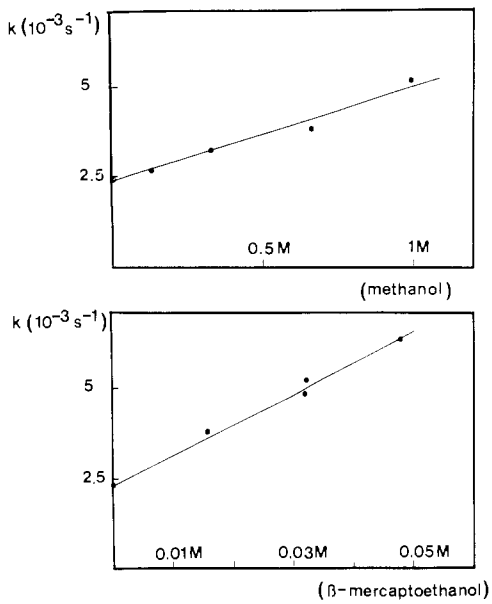


FIGURE 4: Effect of methanol and β-mercaptoethanol on the D-galactal release rate constant. Experimental conditions are as described in Figure 3.

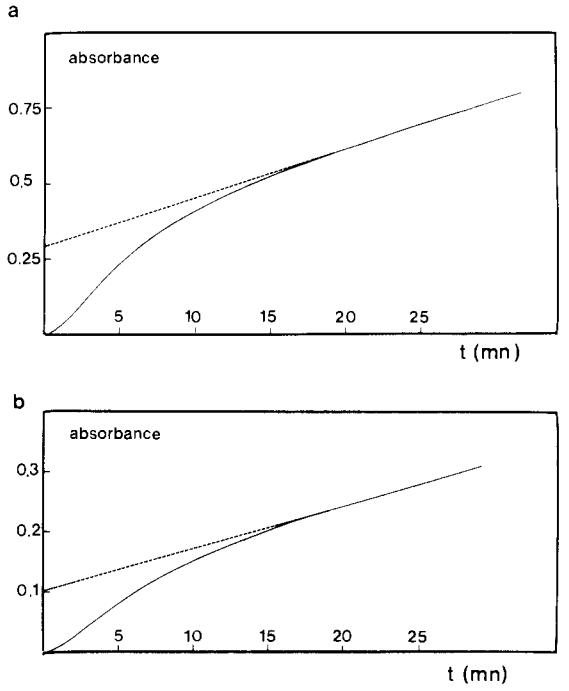


FIGURE 5: Enzymatic hydrolysis of *o*-Np-Gal, in the absence of magnesium, after previous incubation of the enzyme with D-galactal and without (a) or with (b) Mg^{2+} ions. Experimental conditions: a small aliquot of β-galactosidase, incubated with D-galactal in 10 mM EDTA, pH 7.0, and 0.145 M NaCl (a) or 33 mM Tes, pH 7.0, 0.145 M NaCl, and 1 mM $MgSO_4$ (b), was introduced in 3 mL of 4.14 mM *o*-Np-Gal, 0.62 M methanol, 10 mM EDTA, pH 7.0, and 0.145 M NaCl.

Wentworth & Wolfenden (1974). This allows one to calculate the corresponding rate constants.

No fast dissociation occurs, as demonstrated by the absence of initial hydrolysis rate after incubation with an excess of D-galactal. The dissociation of the complex can be analyzed as a single exponential phenomenon, with a k_{off} of 2.5×10^{-3} s^{-1}.

The large effect of nucleophilic compounds (methanol or mercaptoethanol) on this value indicates that this k_{off} value corresponds mainly to the rate constant of hydration of the complex. This was confirmed by the direct determination of the rate of disappearance of D-galactal. These results are in agreement with the conclusion of Wentworth & Wolfenden (1974), obtained by using an enzymatic coupled assay with galactose dehydrogenase. The true dissociation rate of the enzyme–D-galactal complex (as opposed to its hydration) is therefore negligible.

The fact that the release of D-galactal from the enzyme occurs mainly through the chemical transformation of the complex argues for the occurrence, after the binding of D-galactal to the enzyme, of an isomerization step, quite irreversible, which transforms the original Michaelis complex into a second complex, analogous to a 2-deoxygalactosyl enzyme. The existence of the second complex would be demonstrated by a hyperbolic dependence of the k_{on} value upon the substrate concentration. Previous results of Wentworth & Wolfenden (1974) failed to provide evidence of such a saturation. We also have not been able to demonstrate such an isomerization step by kinetic means, even though we worked with higher D-galactal concentrations and with lower *o*-Np-Gal concentrations in order to decrease the *o*-Np-Gal inhibition. However, the very slow value of the k_{on} constant (4.5×10^2 M^{-1} s^{-1}) can hardly be accounted for by a simple bimolecular reaction, and an isomerization step must be involved. To confirm this hypothesis, Deschavanne et al. (1978a) have shown that the presence of D-galactal induces a difference spectra of the enzyme, which is distinctive from the one observed with the classical inhibitor isopropyl thiogalactoside.

Nevertheless, the alternate possibility of the occurrence of a single enzyme–D-galactal complex, with a formation rate constant of 4.5×10^2 M^{-1} s^{-1}, should be considered. Within such a hypothesis, the conformation change of the enzyme would occur simultaneously with the formation of the complex, which could explain the very low association rate constant and the slow (if any) dissociation of this complex. Such an hypothesis suggests that the enzyme would bypass the recognition step that is classically considered as the first step during enzyme catalysis. It therefore appears more unlikely than the existence of an isomerization following the formation of a Michaelis complex. Whatever the mechanism, the inhibition constant for D-galactal, either determined directly or obtained by the k_{off}/k_{on} ratio, is close to 5 μM, which ranks D-galactal among the best known inhibitors of the enzyme.

(II) Study of the Action of D-*Galactal in the Absence of Magnesium. Equilibrium Constant for Interaction between* D-*Galactal and the Enzyme.* β-Galactosidase was incubated with D-galactal, in the presence of 10 mM EDTA, as described under Materials and Methods. Aliquots were introduced into the reaction mixture, in the presence of 1 mM $MgSO_4$. The concentration is large enough to immediately activate the free enzyme (Tenu et al., 1972). Under these conditions, the appearance of *o*-nitrophenol is similar to that obtained after incubating the enzyme and D-galactal in the presence of Mg^{2+} (see Figure 3). From the v_0 and v_∞ values (eq 1), one can calculate a K_I value close to 10 mM.

Kinetic Study of the Binding of D-*Galactal.* When enzyme was introduced into a reaction mixture in the presence of 10 mM EDTA, no significant inhibition occurred in the studied concentration range for D-galactal. Therefore, it was not possible to determine the kinetic binding constant.

Kinetic Study of the Release of D-*Galactal.* When enzyme, incubated with D-galactal and 10 mM EDTA, was introduced into a reaction mixture in the presence of 10 mM EDTA, the appearance of *o*-nitrophenol becomes more complicated (Figure 5a): a slow activation process first occurs, followed by an inactivation one, to reach a steady-state. As a conse-

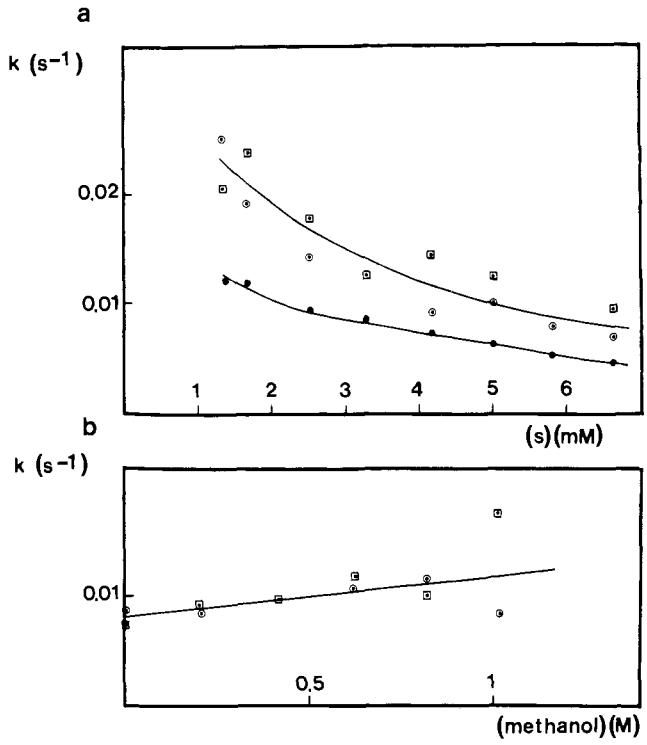


FIGURE 6: Effect of *o*-Np-Gal and methanol concentration on the fast exponential rate constant (see the text). Experimental conditions are as described in parts a (⊙) and b (⊡) of Figure 5. The filled circles (●) refer to the exponential inactivation rate constant when Mg-activated enzyme is introduced in an identical solution [see Tenu et al. (1972)].

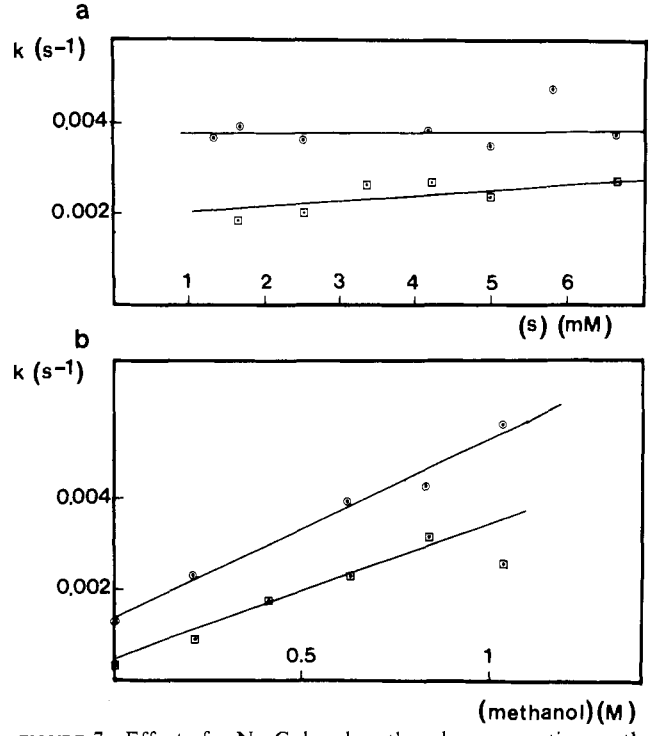


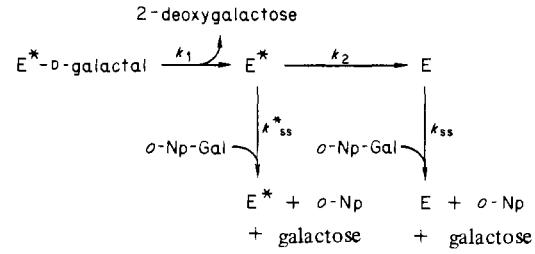
FIGURE 7: Effect of *o*-Np-Gal and methanol concentration on the slow exponential rate constant (see the text). Experimental conditions are as described in Figure 6.

quence, the enzyme exhibits, during the course of the reaction, a higher activity than usual in the presence of EDTA. Analogous phenomena are obtained when the enzyme, previously incubated in the presence of D-galactal and 1 mM Mg^{2+}, is introduced into the reaction mixture with EDTA (Figure 5b). To test such an analogy, we modified two parameters, substrate concentration, which is known to modify the rate of conformational Mg-dependent activation and inactivation (Tenu et al., 1972), and methanol concentration, which increases the rate of release of D-galactal (see above). In each experiment, the data could be analyzed according to eq 2, and the two exponential constants could be determined. The results are shown in Figures 6 and 7. As a comparison, the inactivation rate constant corresponding to the isomerization of Mg-activated enzyme to Mg-free enzyme (experiments carried out in the absence of D-galactal) has been determined under the same conditions of substrate and methanol concentrations. The data are shown in Figure 6.

Discussion. In the absence of magnesium, the interaction of β-galactosidase with D-galactal is very different from that observed in the presence of magnesium. First, the K_I value becomes 10 mM, which represents an increase of 2000-fold, although, with classical substrates or inhibitors, the K_s or K_I values change by no more than 1 order of magnitude when magnesium ions are removed.

This large variation arises from a considerable decrease of the binding constant of D-galactal; the Mg-free enzyme exhibits a lower ability to form a D-galactal–enzyme complex. Furthermore, when this complex is formed, the release of free enzyme, as observed by diluting the complex into a large excess of substrate solution in the presence of EDTA and testing the activity of the free enzyme, is not classical; the enzyme exhibits a higher activity than that usually obtained in Mg-free solutions and then isomerizes slowly to reach its classical activity.

Scheme I



This process is described in Scheme I, in which E* stands for the conformational state of the enzyme obtained in the presence of D-galactal, E stands for the conformational state obtained with EDTA, k_1 is the catalytic constant of formation of 2-deoxygalactose, k_2 is the isomerization constant from the more active to the less active states, and k^*_{ss} and k_{ss} are the steady-state rates with which *o*-Np-Gal is hydrolyzed by 1 mol of E* and E, respectively.

According to this scheme, the appearance of the product is given by

$$P = k_{ss}E_t t + \frac{E_t - E_0}{(k_1 - k_2)k_1}(k^*_{ss}k_1 - k_{ss}k_2)(1 - e^{-k_1 t}) + \frac{E_t - E_0}{(k_2 - k_1)}\frac{k_1}{k_2}(k_{ss} - k^*_{ss})(1 - e^{-k_2 t}) \quad (4)$$

where E_t and E_0 stand respectively for the total enzyme and the enzyme present as free form at zero time.

The experimental data were analyzed according to eq 2 (see Materials and Methods), and two exponential constants, k_A and k_B, were determined. One corresponds to the faster exponential phenomenon and the other one corresponds to the slower one. However, comparative inspection of eq 2 and 4 shows that it is not possible to determine which one, k_A or k_B, is related to k_1 or k_2. In order to obtain this information and to confirm Scheme I, we performed similar experiments with various concentrations of methanol, whose presence must increase the release of E* (k_1 step) and, at various concentrations

of substrate, whose presence should slow down the E* → E isomerization (k_2 step). The results are shown in Figures 6 and 7. Unambiguously, the fast phenomenon can be attributed to an isomerization step, occurring mainly through the free enzyme, while the slower process can be attributed to the release of free enzyme from the D-galactal–enzyme complex.

The nature of the most active transient state could be questioned, especially in regard to its relationship with the Mg-induced conformation. For this reason, similar experiments were performed after incubating the enzyme with D-galactal, in the presence of 1 mM Mg^{2+} ions.

Because of the dependence of the fast rate constant on the methanol and substrate concentration (Figure 6), this rate can be related to the isomerization step. This hypothesis can be directly tested by comparing the rate constant value to the rate of isomerization of the Mg-activated enzyme into the Mg-free enzyme (Figure 6). These values are very similar, and the difference of a factor of 2 can be mainly related to the difficulty of the quantitative analysis of the data, which probably causes the discrepancy between the slow rate constant and the k_{off} value previously determined.

The two isomerization rate constants determined after incubation of the enzyme with D-galactal in the presence and in the absence of Mg^{2+} ions are identical, and it can therefore be concluded that similar conformations are obtained in both cases.

Conclusion

The nonclassical behavior of β-galactosidase with D-galactal, as described by Lee (1969), can be explained by the values of the rate constants involved in this interaction; both the binding and the release rate constants are surprisingly low.

The bimolecular binding constant, with a value of 4.5×10^2 M^{-1} s^{-1}, is more probably accounted for by an isomerization step. This isomerization can be considered mainly as irreversible in that the intermediate cannot reverse to the initial compounds (D-galactal and enzyme) with a significant rate constant but must undergo reaction until the release of 2-deoxygalactose. A plausible explanation implies that the formation of the intermediate involves the opening of the double bond present in D-galactal to form a 2-deoxygalactosyl enzyme. But, as evidenced by the difference spectrum observed in the presence of D-galactal, this isomerization step not only involves a rearrangement of the substrate but also modifies the conformation of the enzyme. It does not appear possible to determine if these two phenomena occur simultaneously or if one is the consequence of the other. However, by analogy with what is observed with aryl galactosides, it can be suggested that the conformational change occurs first, enabling the chemical reaction to proceed.

Once this intermediate is formed, its hydration occurs very slowly with the rate constant close to that of the hydrolysis of acyl glycosides (Brown & Bruice, 1973), which could suggest that no catalytic group of the enzyme is involved in this hydration step. Such a hypothesis is confirmed by the observation of Deschavanne (1976) with PO 13 mutant enzyme. The kinetic constants of this enzyme are all different from those of the wild-type enzyme (Deschavanne et al., 1978b); thus, the k_{on} value of binding of D-galactal is 0.42 M^{-1} s^{-1}. However, its k_{off} value (2.4×10^{-3} s^{-1}) is identical with the one we observe.

The experiments carried out in the absence of magnesium strengthen the hypothesis derived from the study of the interaction in the presence of magnesium. The Michaelis constant for D-galactal increases by a factor of at least 2×10^3 when the enzyme exists in the Mg-free conformation. No binding rate constant can be determined, and the large decrease of affinity that we observe probably does not originate in a decrease of the dissociation constant of the Michaelis complex (the other substrates or inhibitors of the enzyme exhibit a difference of a factor of 10 or less) but in a large decrease of the isomerization rate. Whatever the change involved in this step, the Mg-activated enzyme can undergo it with more efficiency than the Mg-free enzyme. This behavior can be compared to the enzymatic hydrolysis of aryl galactosides in the absence of magnesium wherein no conformational change can be evidenced during the catalysis and the reaction can occur without a compulsory conformational change. On the other hand, such a conformational change appears to be critical in the reaction with D-galactal, since it has been shown that, after the catalysis, the enzyme is released in a conformation different from the original one.

Once the intermediate is formed, with its associated conformational change, the enzyme can isomerize to its former state only after the release of the product. When the enzyme is incubated in the presence of D-galactal, this isomerization occurs faster than the rate of reaction with D-galactal (see extrapolation of the data of Figure 6 in the absence of substrate) and cannot be evidenced. But, when the liberation of 2-deoxygalactose occurs in the presence of an excess of another substrate (*o*-Np-Gal), the binding of this substrate slows down the isomerization of the enzyme, thus emphasizing the transitory appearance of a more active state of the enzyme. The rate constant of this isomerization is comparable to the one observed for the isomerization of the enzyme from the Mg-activated state to the Mg-free one, pointing out the similarity (if not identity) of the conformational state of the enzyme in the Mg-activated form and of the D-galactal–enzyme complex.

This new conformation could be the consequence of the D-galactal binding and could occur previously or concomitantly to the chemical reaction between the enzyme and the D-galactal molecule. An alternative explanation would involve a preexisting equilibrium of the enzyme between two states, E and E*. The decrease by a factor of at least 10^3 in the affinity of β-galactosidase for D-galactal indicates that the equilibrium would be largely in favor of E. By binding only to the E* form, D-galactal would displace the equilibrium toward E*, while *o*-Np-Gal, binding to the two forms with comparable affinity, would allow the equilibrium to return toward E. These two models differ by the sequence of the two kinetic events, the binding of D-galactal, and the isomerization. They both underline the importance of dynamic interaction in β-galactosidase catalysis and the positive influence of Mg^{2+} ions for such interactions.

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Role of Mixed Oxidation States in the Oxidation of Hemerythrin Species by Ferricyanide Ion†

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ABSTRACT: The oxidation of deoxy- and oxyhemerythrin to methemerythrin by $Fe(CN)_6^{3-}$ has been examined. The first step produces $(semi\text{-}met)_O$, a half-oxidized form of hemerythrin which has different spectral and kinetic properties from $(semi\text{-}met)_R$ which is produced by one-electron reduction of methemerythrin. Further oxidation of $(semi\text{-}met)_O$ to methemerythrin usually occurs only indirectly via disproportionation of $(semi\text{-}met)_O$ to met and deoxy forms within the octameric framework. Oxidation of $(semi\text{-}met)_R$ by $Fe(CN)_6^{3-}$ is a direct second-order reaction. $(Semi\text{-}met)_O$ is reduced rapidly by dithionite to deoxy, whereas that of $(semi\text{-}met)_R$ is a slow biphasic process. The oxidation of oxyhemerythrin occurs via the deoxy species, little, if any, reactivity being attributable to the oxy form. The oxidation of the azide adduct of $(semi\text{-}met)_R$ is biphasic, in which in step one N_3^- is removed during the oxidation to methemerythrin, which in the second step recombines with N_3^-. Rate parameters for all these processes at pH 8.2 and 6.3 for protein from *Phascolopsis gouldii* and *Themiste zostericola* have been obtained. The implications of these findings to hemerythrin chemistry are discussed.

Hemerythrin (Hr) occurs in the erythrocytes of certain marine worms in a polymeric, usually octameric, form. It is an interesting example of a respiratory protein which contains two linked nonheme irons in each subunit (Hendrickson, 1978; Kurtz et al., 1977; Loehr & Loehr, 1979; Stenkamp & Jensen, 1979). When both irons are in oxidation state +2, this (deoxy) form interacts reversibly with oxygen to give the oxy form. Both deoxy and oxy forms are easily oxidized [usually $Fe(CN)_6^{3-}$ is employed] to a met species containing irons only in the oxidation state +3. This is no longer O_2 sensitive but does react with a number of anions to form adducts with a wide range of stabilities (Keresztes-Nagy & Klotz, 1965; Meloon & Wilkins, 1976; Olivas et al., 1979).

Apart from its importance in preparative hemerythrin chemistry, a study of the oxidation of hemerythrin species to methemerythrin by $Fe(CN)_6^{3-}$ is of interest for several reasons. (a) The reaction of deoxy- and oxyhemerythrin with the iron(III)–cyanide complex is a noncomplementary redox reaction, involving a one-electron oxidant and a two-electron reductant. The role in the oxidation of the half-oxidized form of the protein which we shall term $(semi\text{-}met)_O$, in which one of the binuclear irons is +3 and the other is +2, can therefore be delineated. Furthermore, the results can be compared with those obtained by ferricyanide oxidation of a semi-met form which has been recently obtained by a one-electron reduction of the met form using dithionite (Harrington et al., 1978) and which we now term $(semi\text{-}met)_R$. (b) A comparison of the kinetic behavior of the deoxy and oxy forms will determine whether oxidation of the latter occurs directly or via the deoxy form with which it is in dissociative equilibrium. (c) Finally, the observation of further absorption changes following rapid oxidation of hemerythrin species is a distinct possibility since the geometries of, and even the nature of, the ligands attached to the iron sites in the iron(II) forms are likely to be quite different from those in the iron(III) species (Kurtz et al., 1977). Conformational adjustments will be necessary following the oxidation, and these might be reflected in spectral changes.

Apart from some interesting qualitative observations (Brunori et al., 1971), there have been no reported kinetics of oxidation of hemerythrin species by $Fe(CN)_6^{3-}$ and we describe a detailed study at pH 8.2 and 6.3 of this protein from two sipunculids, *Phascolopsis gouldii* and *Themiste zostericola*.[1] These are pH values for which we have previous data, and they are near the limits of the range of pH which can be conveniently studied with this protein.

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[1] This species obtained from Pacific Biomarine Supply was formerly considered to be *Themiste pyroides* and was so designated in previous publications by us and others (Gormley et al., 1978).

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