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THE BINDING OF A FLUORESCENT ACTIVATOR 2-(*N*-DECYL)AMINONAPHTHALENE-6-SULFONIC ACID TO PYRUVATE OXIDASE

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Summary

E. coli pyruvate oxidase (pyruvate:ferricytochrome b_1 oxidoreductase, EC 1.2.2.2) is a peripheral membrane flavoenzyme which has been purified to homogeneity. In vivo the oxidase resides on the inner surface of the cytoplasmic membrane and is coupled to the bacterial electron transport chain. In vitro, the purified oxidase requires lipids for full enzymatic activity. Previous studies have characterized the conformational and energetic coupling between the lipid-binding site(s) and the catalytic active site. The affinity of the enzyme for phospholipids and detergents is significantly enhanced when the flavoprotein is in the reduced form, i.e., in the presence of pyruvate and the required cofactor, thiamin pyrophosphate. The lipid-binding studies were hindered due to the complicating factor of the self-association of the substrate-reduced flavoprotein. In this paper, fluorescence techniques are employed to measure the binding of a detergent-like activator to the oxidase. The experiments are performed at much lower protein concentrations than previously employed, so that protein aggregation is not a problem. The chromophore on the activator, 2-(*N*-decyl)aminonaphthalene-6-sulfonic acid is effective at quenching the pyruvate oxidase intrinsic tryptophan fluorescence. Quenching titrations are used to obtain the binding isotherm.

At DNS concentrations less than 10^{-5} M, the results show a larger amount of DNS binding to the reduced flavoprotein than to the oxidized form of the enzyme. This is the concentration range where DNS is an effective activator of the enzyme. This represents a class of binding sites specifically found on pyru-

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Abbreviation: Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); DNS, 2-(*N*-decyl)aminonaphthalene-6-sulfonic acid; DCIP, 2,6-dichloroindophenol.

vate oxidase and not apparent in other proteins such as lysozyme or aldolase. At the DNS concentration which is optimum for activation approx. 20 molecules of DNS are bound per enzyme tetramer in the absence of the substrate. The pyruvate-reduced form of the enzyme binds about 40—50 molecules of DNS per tetramer. Qualitatively, the results are similar to what was previously found for both sodium dodecyl sulfate and cetyl trimethylammonium bromide. However, in both these cases, the amount of bound detergent was nearly an order of magnitude less than the values obtained using DNS.

Introduction

Pyruvate oxidase (pyruvate:ferricytochrome b_1 oxidoreductase, EC 1.2.2.2) is a peripheral membrane enzyme that can be purified without the aid of detergents from *Escherichia coli* O'Brien et al. [1]. This enzyme catalyzes the oxidative decarboxylation of pyruvate to acetic acid and CO_2. Investigations using Kaback vesicles have shown that the oxidase is bound to the inner surface of the cytoplasmic membrane of *E. coli* [2], where it is coupled to the electron transport chain [3] and may be involved in driving solute active transport. Previous characterization of the enzyme has shown that it is an α_4 tetramer of 60 000 dalton subunits. Each subunit contains a non-covalently bound FAD [4,1,20]. A second cofactor, thiamin pyrophosphate, is also required for enzymatic activity in the form of a divalent cation complex [4,5]. A most interesting aspect of this enzyme is the dramatic effect of lipids and detergents on the catalytic activity [6,7]. A wide range of amphipaths including the detergents SDS and cetyl trimethylammonium bromide as well as various phospholipid vesicles are capable of stimulating V of the in vitro steady-state assay by about 25-fold. In addition, the presence of these amphipaths modulates the binding interactions between the enzyme and the ligands involved in catalysis, pyruvate and thiamin pyrophosphate [5]. It has been demonstrated that the presence of these catalytic ligands influences the binding of detergents [8] and phospholipids [9] to the enzyme. In the presence of both pyruvate and thiamin pyrophosphate, pyruvate oxidase manifests an enhanced affinity for phospholipids and detergents. The key chemical event associated with this enhanced lipid binding appears to be the chemical reduction of the protein bound flavin. The reduced form of the flavoprotein not only binds to lipids with enhanced affinity but also tends to self-aggregate at high protein concentrations. The self-aggregation of the reduced form of pyruvate oxidase has restricted the quantitative interpretation of all the previously reported lipid and detergent binding studies with this enzyme. In this paper, fluorescent techniques are used to quantitate the binding of an amphipathic activator of pyruvate oxidase. The sensitivity of the technique permits the experiments to be done at very low protein concentrations essentially equivalent to steady state assay conditions. It has previously been shown [19], that pyruvate oxidase does not self-aggregate under these conditions.

Materials and Methods

Reagents. Aldolase, bovine serum albumin, lysozyme, Pipes, DCIP, sodium pyruvate and thiamin pyrophosphate were all obtained from Sigma. The

sodium pyruvate and Pipes buffer were filtered through activated charcoal (Fischer) to eliminate fluorescent impurities. Sodium ferricyanide was obtained from K & K Laboratories. DNS was obtained from Molecular Probes. All other chemicals were reagent grade. Deionized, glass-distilled water was used for all solutions.

Pyruvate oxidase. Details of the enzyme purification and characterization have been described elsewhere [1]. Enzyme activity was measured using a ferricyanide reductase assay [10] or a dichloroindophenol reductase assay [11].

2-(N-Decyl)Aminonaphthalene-6-sulfonic acid binding to pyruvate oxidase. When the fluorescent activator DNS interacts with pyruvate oxidase, the intrinsic fluorescence of the protein can be quenched by more than 75%. This fluorescence quenching was used to measure the binding isotherm of DNS to the enzyme at room temperature (24° C). Small aliquots of solutions of DNS (0.001 M and 0.01 M solutions in methanol) were successively added to a solution of pyruvate oxidase 3 ml 0.1 M Pipes buffer, pH 5.7, in a fluorescence cuvette. Careful mixing and a 10 min incubation period followed each addition. The intrinsic fluorescence of the protein at 330 nm was measured relative to a blank that contained equivalent amounts of DNS but no protein. The excitation wavelength used was 273 nm, which corresponds to a minimum in the DNS excitation spectrum. All fluorescence measurements were obtained with a Perkin Elmer MPF-44A fluorimeter equipped with a circulating constant-temperature bath maintained at 24° C. Excitation and emission bandwidths were 5 nm. All fluorescent intensities were corrected for DNS inner filter effects and volume changes. In the presence of 20 μM thiamin pyrophosphate, 50 mM $MgCl_2$ and 10 mM pyruvate, the absorption at 273 nm was about 0.4. This absorbance remains constant throughout the titration of pyruvate oxidase under these conditions. All fluorescence quenching data are reported relative to the initial protein fluorescence in the absence of DNS.

The fluorescence titration data were analyzed by a method similar to the one developed by Green [12], and Holbrook [13] and Hardwicke [14]. The method involves measurement of the titration curve at various protein concentrations. It is assumed that the extent of fluorescence quenching is a monotonic function of the amount of DNS bound to the protein. The basic equation [15] is:

$$\frac{1}{P_t} = \frac{1}{L} \cdot \frac{L_t}{P_t} - \frac{\bar{\nu}}{L} \tag{1}$$

P_t is the total subunit concentration. L_t and L are the total and free DNS concentrations respectively, and $\bar{\nu}$ is the number of moles of DNS bound per mol of enzyme subunit. This is just an equation of mass balance. Fluorescence quenching is measured using a series of solutions with varying values of L_t and P_t. It is assumed that in solutions with a common value for the fractional quenching that the value of $\bar{\nu}$ is the same. For each set of solutions manifesting the same extent of fluorescence quenching a plot is constructed of $1/P_t$ vs. L_t/P_t. The slope and intercept of the resulting straight line yields the values of the free ligand concentration [L] and the amount of DNS bound per enzyme subunit [ν]. In this way a binding isotherm can be constructed.

Results

The uncorrected excitation and emission spectra of pyruvate oxidase and DNS in aqueous solutions are shown in Fig. 1. Two features are worth noting. First there is good overlap between the protein emission peak and the DNS excitation (absorption) peak. Second, the minimum at 273 nm in the DNS excitation spectrum permits excitation of the enzyme with minimum interference from the fluorescence from the amphiphile.

It was hoped that DNS would activate pyruvate oxidase in a manner similar to that observed with sodium dodecyl sulfate. That this seems to be the case is shown in Fig. 2A. At $1 \cdot 10^{-5}$ M DNS the specific activity of pyruvate oxidase is increased approx. 18-fold. The fluorescent amphiphile appears to be monomeric and not micellar at these concentrations. Because of the favorable spectral overlap DNS is an effective quencher for tryptophan. As a test, the fluorescence quenching by DNS of bovine serum albumin fluorescence was investigated. Fig. 3 shows the fluorescence quenching titration curve obtained. At $1 \cdot 10^{-5}$ M DNS the fluorescence emission from the protein is completely abolished. The apparent dissociation constant of DNS to bovine serum albumin obtained from this titration curve is $7.2 \cdot 10^{-7}$ M. This value is in good agreement with the dissociation constant for sodium dodecyl sulfate binding to high affinity sites on bovine serum albumin $9.1 \cdot 10^{-7}$ M [16].

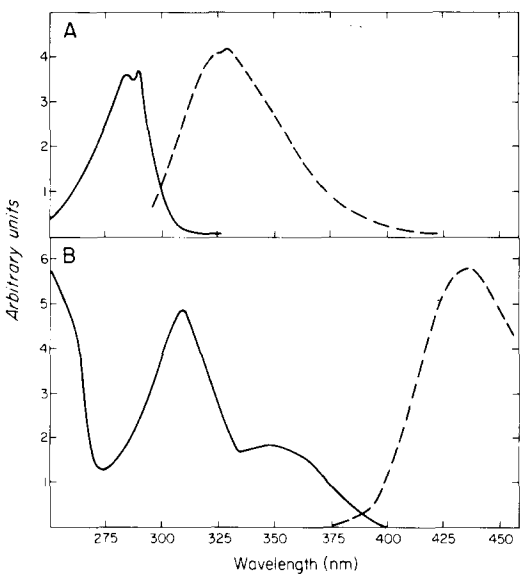

Fig. 1. Excitation and emission spectra of pyruvate oxidase and DNS in 0.1 M Pipes buffer, pH 5.7 at 24° C. All spectra are uncorrected. Both the excitation and emission bandwidths were 5 nm. A. Excitation spectrum of the oxidized form of pyruvate oxidase, monitored at 330 nm (———). Emission spectrum of the enzyme using an excitation wavelength of 284 nm (- - - - - -). The intensity of the emission spectrum was reduced by a factor of 2/3 when the flavin was reduced by pyruvate; however, the shape of the spectrum is not significantly altered. The decrease in emission intensity is due to the absorbance of the ligands thiamin pyrophosphate and pyruvate. B. Excitation spectrum of DNS fluorescence monitored at 430 nm (———). Emission spectrum of DNS using an excitation wavelength of 310 nm (- - - - - -).

Fluorescence quenching titration curves by DNS are shown in Fig. 4 for three different concentrations of pyruvate oxidase ranging from 12 μg/ml to 96 μg/ml. The data are plotted as a function of log (L_t/P_t) for convenience. Data points from the three titration curves shown in Fig. 4 corresponding to identical extents of quenching are used to construct the plot shown in Fig. 5. As expected from Eqn. 1, the data obtained for each value of the extent of fluorescence quenching yield a straight line. From the slopes and intercepts of these lines are obtained the values of the free ligand concentration and the number of DNS molecules bound per pyruvate oxidase subunit for each value for fluorescence quenching. The binding isotherm of DNS to oxidized pyruvate oxidase is shown in Fig. 2B. The extent of fluorescence quenching as a function of the number of DNS molecules bound is plotted in Fig. 6. Similar data are

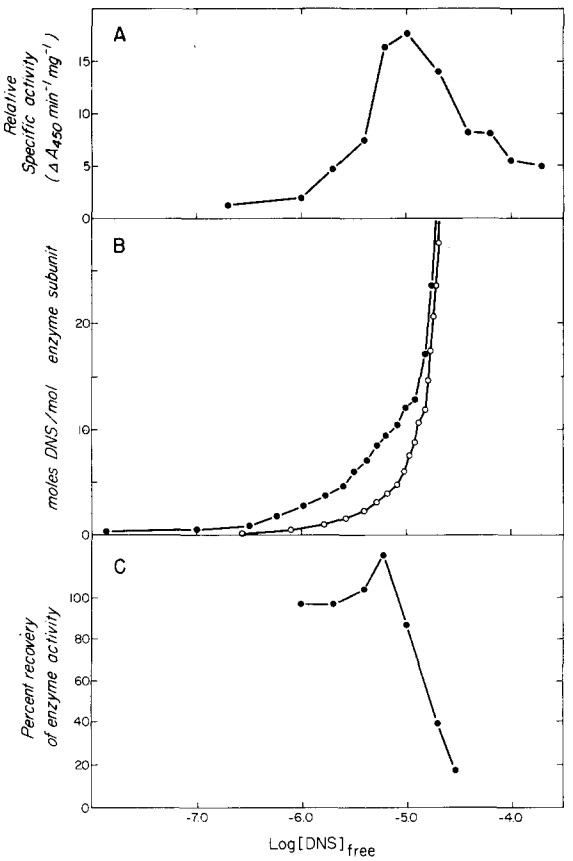

Fig. 2. The interaction of DNS with pyruvate oxidase. A. Activation of pyruvate oxidase at 24°C by DNS. The ferricyanide reductase assay was used. Small aliquots of DNS in methanol were added to activate the enzyme. The methanol did not affect the activity of the enzyme. B. Binding of DNS to pyruvate oxidase at 24°C. Open circles (○———○) show the binding of DNS to the oxidized form of the enzyme in 0.1 M Pipes buffer, pH 5.7. Closed circles (●———●) represent the binding of DNS to the pyruvate-reduced oxidase in 0.1 M Pipes buffer, pH 5.7, containing 20 μM thiamin pyrophosphate, 50 mM $MgCl_2$ and 10 mM sodium pyruvate. C. Pyruvate oxidase activity recovered after the fluorescence titration experiment with the pyruvate-reduced enzyme. The percent recovery of activity relative to an identical sample of the enzyme without DNS, is shown. The control sample retained 60% of the original specific activity. Each experiment requires about 2—3 h.

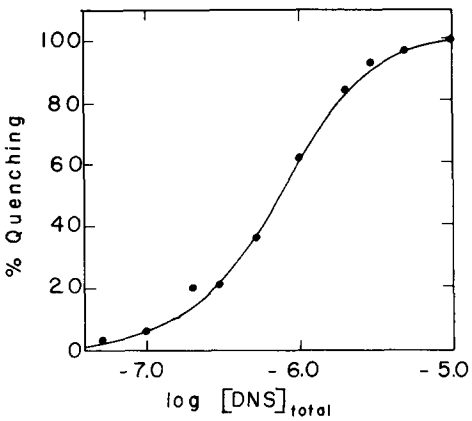

Fig. 3. DNS quenching of the protein fluorescence of bovine serum albumin. Aliquots of DNS in methanol were added to 1 μM solutions of bovine serum albumin in 0.1 M Pipes buffer, pH. 5.7. The excitation and emission wavelengths were 273 and 342 nm, respectively. Both bandwidths were 5 nm. All of the protein fluorescence was quenched in the presence of 10 μM DNS.

also shown for the substrate-reduced form of pyruvate oxidase. Experiments with the reduced form of pyruvate oxidase were performed by including 20 μM thiamin pyrophosphate, 50 mM $MgCl_2$ and 10 mM pyruvate in the buffer solution. Data with reduced pyruvate oxidase similar to those shown in Figs. 4 and 5 were obtained using protein concentrations ranging from 6 to 24 μg/ml (data not shown). The binding isotherm and the quenching curve for reduced pyruvate oxidase are shown in Figs. 2B and 6, respectively. Fig. 6 shows that the extent of fluorescence quenching as a function of the amount of bound DNS is quite similar for both the oxidized and reduced forms of pyruvate oxidase. However, Fig. 2B shows that in the presence of pyruvate and thiamin pyrophosphate the enzyme binds significantly more amphiphile than in the

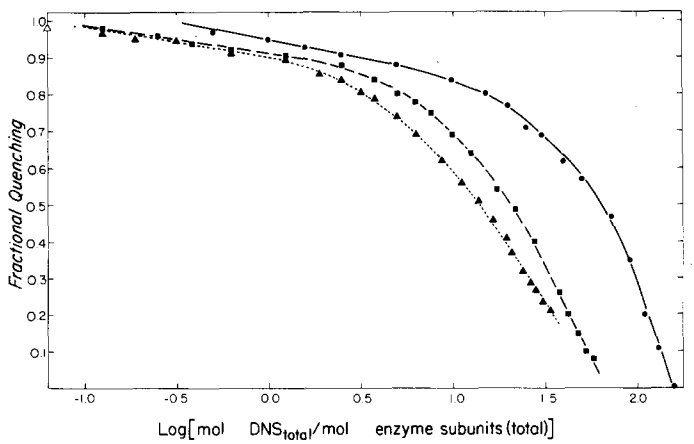

Fig. 4. Fluorescence quenching by DNS of the oxidized form of pyruvate oxidase in 0.1 M Pipes buffer, pH 5.7: ●———●, 0.2 μM enzyme subunits (12 μg/ml); ■———■, 0.8 μM enzyme subunits and; ▲———▲, 1.6 μM enzyme subunits. The experiment was performed as described in the text. Each titration was performed three times and the results were averaged.

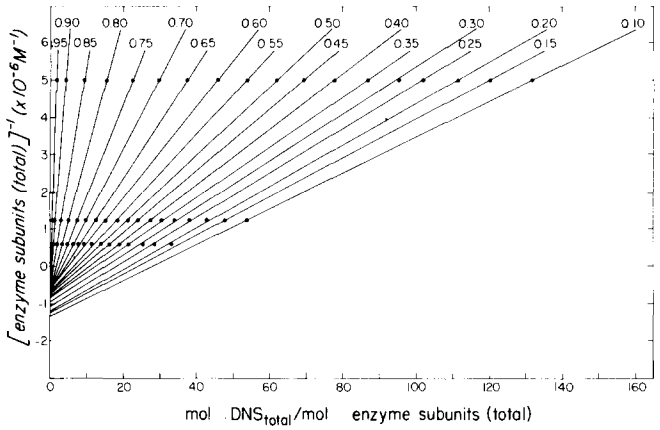

Fig. 5. Graphical analysis of the DNS titration data for the oxidized form of pyruvate oxidase. Each line represents a set of data at the indicated value of the fractional quenching, taken from Fig. 4. The numbers above each line correspond to the fractional quenching values that were used for that line. The data were analyzed as described in the text. The slope and intercept of each line is used to obtain the free DNS concentration [L], and the amount of DNS bound [ν] corresponding to the extent of the fluorescence quenching observed.

absence of these ligands in the DNS concentration range at which the amphiphile is activating. This is qualitatively similar to that which has been previously reported to be the case with sodium dodecyl sulfate and cetyl trimethylammonium bromide. Fig. 2C demonstrates that the recovery of the enzyme activity is excellent at the end of the fluorescence titration experiments in the DNS concentration range of interest. At concentrations of DNS greater than about $1 \cdot 10^{-5}$ M, it is clear that large amounts of amphiphile bind to pyruvate oxidase and the enzyme is denatured.

As additional controls the effect of DNS on the fluorescence emission of aldolase and lysozyme were also examined. In both cases DNS was shown to quench the fluorescence at concentrations above $1 \cdot 10^{-5}$ M and little effect was seen below this concentration.

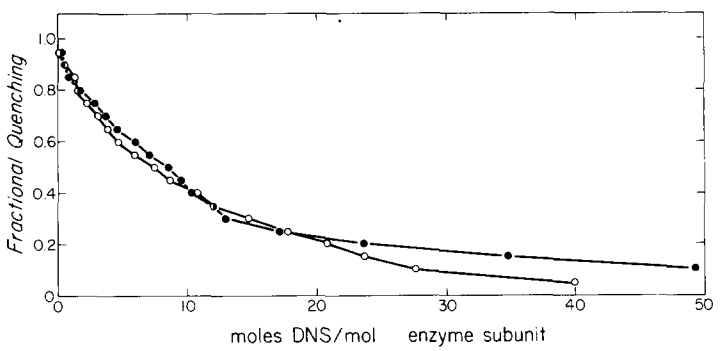

Fig. 6. Fluorescence quenching of the oxidized (○———○) and the substrate-reduced (●———●) forms of pyruvate oxidase as a function of the mol DNS bound per mol of enzyme subunit.

Discussion

Pyruvate oxidase is one of a number of membrane enzymes which is lipid-stimulated or dependent on lipids for enzymatic activity [17,18]. In the presence of any one of a number of amphiphiles, the specific activity of pyruvate oxidase is greatly enhanced. Charged detergents such as sodium dodecyl sulfate activate pyruvate oxidase below their critical micelle concentrations. Previous reports have demonstrated a conformational and energetic coupling between the catalytic active site on pyruvate oxidase and lipid binding sites [5,8]. One manifestation of this is that the affinity of the protein for both charged detergents and phospholipid vesicles is greatly enhanced in the presence of the substrate and cofactor. The major purpose of this paper is to quantitate further this relationship by measuring the binding of a fluorescent amphiphile under conditions in which protein self-aggregation is not a complicating factor. The quenching of the intrinsic fluorescence from pyruvate oxidase by DNS was used to monitor DNS binding at very low protein concentrations with both the oxidized and reduced forms of pyruvate oxidase. Experiments with pyruvate oxidase in the presence of pyruvate and thiamin pyrophosphate were all performed using protein concentrations less than 25 μg/ml. Previous studies both with sodium dodecyl sulfate and with dipalmitoyl phosphatidylcholine were all carried out using protein concentrations between 10- and 100-times this value [8]. At the high protein concentrations the formation of lipid protein co-aggregates and co-precipitates is a major problem.

The results of these experiments are summarized in Fig. 2. In the absence of pyruvate and thiamin pyrophosphate, the oxidized flavoprotein manifests high-affinity binding sites for the detergent at detergent concentrations below $1 \cdot 10^{-5}$ M. At $1 \cdot 10^{-5}$ M DNS, the optimum for activation, approx. 5 molecules of DNS are bound per enzyme subunit. Above $1 \cdot 10^{-5}$ M DNS a highly cooperative binding of the detergent to the protein appears to occur. This corresponds to the decrease in specific activity of the enzyme and its apparent denaturation. Below $1 \cdot 10^{-5}$ M the enzyme appears to be stabilized by the presence of DNS.

The interaction between DNS and the pyruvate reduced form of pyruvate oxidase is quite distinct compared to the binding of the oxidized form of the flavoprotein. The differences observed (Fig. 2B) are primarily below DNS concentrations of $1 \cdot 10^{-5}$ M. The binding isotherm appears to be shifted to lower concentrations of DNS and significantly more amphiphile is bound to pyruvate oxidase at low DNS concentrations. Binding of DNS to pyruvate oxidase at concentrations above $1 \cdot 10^{-5}$ M appears to be quite similar for both forms of the enzyme. This same pattern was observed in previous studies with sodium dodecyl sulfate [8]. However, in these earlier experiments the problem of protein aggregation was significant and the protein/detergent solution became turbid.

Although the results obtained with DNS are qualitatively similar to those obtained with sodium dodecyl sulfate, there are significant quantitative differences. In the case of sodium dodecyl sulfate a maximum of a one or two detergent molecules was determined to be bound to each pyruvate oxidase subunit at the optimum concentration for enzyme activation. By comparison, at

$1 \cdot 10^{-5}$ M DNS there appear to be 10—12 molecules of DNS bound per enzyme subunit. Since the enzyme remains tetrameric under these conditions [19], approx. 40 molecules of DNS are bound per pyruvate oxidase molecule under steady state assay conditions when the enzyme is maximally activated. It is not clear whether the earlier reported results with sodium dodecyl sulfate were low due to the complication of protein aggregation, or whether DNS is just behaving differently from sodium dodecyl sulfate. In any event, these results suggest that DNS may be forming a micelle-like structure about a hydrophobic region of pyruvate oxidase and that formation of this structure results in activation of the enzyme. At concentrations of DNS above $1 \cdot 10^{-5}$ M considerably larger amounts of detergent bind to the oxidase, resulting in denaturation.

The lipid-binding portion of pyruvate oxidase is thought to be located near the C-terminus. Attempts are presently being made to isolate this portion of the molecule. It is hoped that future studies both with the intact enzyme and with isolated peptides will serve to clarify the structural basis for the lipid activation process.

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