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IM-2 ([2R,3R,1’R]-2-(1’-hydroxybutyl)-3-(hydroxymethyl)butanolide] of Streptomyces sp. strain FRI-5 is one of the butyrolactone autoregulators of Streptomyces species and triggers production of blue pigment as well as the nucleoside antibiotics showdomycin and minimycin. A tritium-labeled IM-2 analogue, 2-3-trans-2(1’-β-hydroxy-[4’,5’-3H]pentyl)-3-(hydroxymethyl)butanolide ([3H]IM-2-C5; 40 Ci/mmol), was synthesized for a competitive binding assay, and an IM-2-specific binding protein was found to be present in the crude cell extract of Streptomyces sp. strain FRI-5. During cultivation for 24 h, the specific IM-2-binding activity increased rapidly, reached a plateau at 10 to 14 h, and declined sharply thereafter, showing only 6% activity after 24 h of cultivation. A Scatchard plot of the binding data demonstrated that the dissociation constant (Kd) for [3H]IM-2-C5 was 1.3 nM, while the Kd for a 3-H-labeled virginiae butanolide (VB) analogue, 2-(1’-α-hydroxy-[6,7,8,9,10,11,12,13]-heptyl)-3-(hydroxymethyl)butanolide ([3H]VB-C7), another butyrolactone autoregulator possessing the opposite configuration at C-1’ was 35 nM. Furthermore, at a 15-fold molar excess, the effectiveness of several autoregulators as nonlabeled competitive ligands against [3H]IM-2-C5 was IM-2 type > VB-C type >> A-factor type, indicating that the binding protein in Streptomyces sp. strain FRI-5 is highly specific toward IM-2. Ultracentrifugation showed that the IM-2-binding protein is present almost exclusively in the 100,000 × g supernatant fraction, indicating that the binding protein is a cytoplasmic soluble protein. The binding protein was purified by ammonium sulfate precipitation, DEAE-Sephalac chromatography, Sephacryl S-100 HR gel filtration, DEAE-SPW high-performance liquid chromatography (HPLC), and phenyl-SPW HPLC. The apparent Mw of the native IM-2-binding protein as determined by molecular sieve HPLC was about 60,000 in the presence of 0.5, 0.3, or 0.1 M KCl, while by sodium dodecyl sulfate-polyacrylamide gel electrophoresis it was about 27,000, suggesting that the native binding protein is present in the form of a dimer.

MATERIALS AND METHODS

**Bacterial strain and culture conditions.** Streptomyces sp. strain FRI-5 (MAFF 10-06015; National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan) (4) was used throughout this study as a source of IM-2-binding protein. The strain was grown at 28°C in a medium containing, per liter, 7.5 g of yeast extract (Difco), 7.5 g of glycerol, and 1.25 g of NaCl (pH 6.5). For the purification of IM-2-binding protein, 2-liter-Sagakuchi flasks containing 500 ml of the medium were inoculated with 15 ml of a 24-h culture and incubated for 12 h at 28°C. Typical yields of the mycelia were about 17 g of wet cells per liter of medium.

**Chemicals.** All the chemicals were of reagent or high-performance liquid chromatography (HPLC) grade and were purchased from either Nacali Tate, Inc. (Osaka, Japan); Takara Shuzo Co. (Shiga, Japan); or Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Marker proteins for molecular sieve HPLC and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Oriental Yeast Co., Ltd., and Pharmacia LKB, respectively.

**Synthesis of nonradioactive and radioactive IM-2-C5.** Cold racemic IM-2-C5 was chemically synthesized as described elsewhere (6) by using pentyl aldehyde during aldol condensation. [3H]IM-2-C5, (2-[1’-β-hydroxy-[4’,5’-3H]-
Further purified by DEAE-5PW HPLC (TSKgel DEAE-5PW, 7.5 by 75 mm; deluted with the same buffer at a flow rate of 1.5 ml/min in buffer B containing 0.3 M KCl at a flow rate of 0.5 ml/min. Proteins were monitored by measuring \( A_{280} \) or fluorescence (excitation, 280 nm; emission, 340 nm).

**Determination of molecular weight.** The molecular weight of the IM-2-binding protein was estimated with 50 µl of the most active fractions eluted from Sephacryl S-100 HR column chromatography by gel filtration HPLC (TSK-G2000 SWXL, 7.5 by 90 cm), preequilibrated with buffer B containing 0.5 M KCl, 0.32 M KCl were concentrated by ultrafiltration (UP-20; Advantec Toyo), and then concentrated to 0.5 M KCl at a flow rate of 0.5 ml/min, and proteins were monitored by measuring \( A_{280} \). Fractions were collected every 30 s and assayed for IM-2-binding activity to determine the elution position of the IM-2-binding protein.

SDS-PAGE was performed with a ready-made 4 to 20% gradient gel (Daichi Pure Chemicals Co., Ltd., Tokyo, Japan) by using a mini-gel apparatus (Daiichi.). Samples from phenyl-5PW HPLC were dialyzed against distilled water at 4°C overnight with three changes to remove all traces of ammonium sulfate, lyophilized, and then dissolved in SDS buffer (10) before being applied to SDS-PAGE gels. Pharmacia LKB Mr standards (94,000, 67,000, 43,000, 30,000, and 20,000) were used as markers.

**IM-2-binding assay.** IM-2-binding activity was assayed by measuring the difference in absorbance of 0-10 M (23.3 mM/min for 30 min and then 15 M M/min for 20 min) in buffer B containing 0.3 M KCl at a flow rate of 0.5 ml/min. Proteins were monitored by measuring \( A_{280} \) or fluorescence (excitation, 280 nm; emission, 340 nm).

**Preparation of cell extract.** Mycelium was harvested, resuspended in a fivefold volume of buffer A [0.05 M triethanolamine-HCl, pH 7.0, containing 0.5 M KCl, 5 mM dithiothreitol, and 0.1 mM p-(aminophenyl)anthranilic hydrochloric], and disrupted by sonication as described elsewhere (6). The sonicated suspension was then centrifuged at 28,000 g for 20 min. Solid ammonium sulfate was added to the supernatant with stirring on ice to give 60% saturation. After 30 min, the precipitate was centrifuged at 28,000 × g for 20 min at 4°C and dissolved in 1 volume of buffer A. The crude cell extract was dialyzed for 6 h against a 200-fold volume of buffer A at 4°C.

**Purification of IM-2-binding protein.** Crude cell extract of *Streptomyces* sp. strain FRI-5 (from 100 g of wet mycelia), prepared as described above by sonication, ammonium sulfate precipitation, and resuspension in 1 volume of buffer A, was dialyzed against a 20-fold volume of buffer A overnight at 4°C with three changes. The dialyzed solution was then applied to a DEAE-Sephascel column (5.5 by 22 cm) preequilibrated with buffer B (0.02 M triethanolamine-HCl, pH 7.0, containing 5 mM dithiothreitol). After a wash with a 2-bed volume of buffer B, adsorbed proteins were eluted with a linear gradient of KCl from 0.1 to 0.5 M in 5-bed volumes of buffer B. Pooled active fractions eluted at 0.23 to 0.32 M KCl were concentrated by ultrafiltration (UP-20; Advantec Toyo), applied in 10-ml portions (250 mg of protein) to a gel filtration column (Sephacryl S-100 HR; 2.6 by 90 cm), preequilibrated with buffer B containing 0.5 M KCl, and eluted with the same buffer at a flow rate of 1.5 ml/min. The active fractions were pooled and concentrated by ultrafiltration. The concentrated solution was further purified by DEAE-5PW HPLC (TSKgel DEAE-5PW, 7.5 by 75 mm; Tosoh, Tokyo, Japan) in aliquots of 500 µl per run (3.9 mg of protein). Proteins were eluted with a linear gradient of KCl from 0.1 to 0.5 M (10 mM/min in buffer B at a flow rate of 0.5 ml/min and monitored by measuring \( A_{280} \). An active peak sample was finally purified by hydrophobic HPLC on a phenyl-5PW column (7.5 by 75 mm; Tosoh) with two steps of a linear gradient of ammonium sulfate from 1 to 0 M (23.3 mM/min for 30 min and then 15 M M/min for 20 min) in buffer B containing 0.3 M KCl at a flow rate of 0.5 ml/min. Proteins were monitored by measuring \( A_{280} \) or fluorescence (excitation, 280 nm; emission, 340 nm).

**RESULTS AND DISCUSSION**

**Synthesis of \( ^{3}H \) IM-2.** Because the natural form of IM-2 contains a 1'-hydroxybutyl side chain at C-2, we at first intended to synthesize 2-[(1'-8-hydroxy-3'-butenyl)-3-(hydroxymethyl)butanolide by coupling 3-butenal with compound 2 to introduce 1'H by catalytic reduction (Fig. 2). However, during aldol condensation, the terminal double bond shifted to 2'-.

We then decided to synthesize \( ^{3}H \) IM-2-C5 in which the C-2 side chain is one carbon longer than in natural IM-2. IM-2-C5 was found to be as effective as the natural compound in triggering blue pigment production in *Streptomyces* sp. strain FRI-5 (16). During the synthesis of compound 3a (Fig. 2), no shift of the terminal double bond occurred, and the resulting \( ^{3}H \) IM-2-C5 gave a very high specific radioactivity of 40 Ci/mmol. Chemical and radiochemical purity was confirmed by reverse-phase C18 HPLC with a radioisotope detector (data not shown).

**Existence of IM-2-binding protein and its profile during the cultivation period.** By using \( ^{3}H \) IM-2-C5 in the presence and absence of a 1,500-fold molar excess of cold IM-2-C5, binding activity in the crude cell extract was measured by equilibrium dialysis. Preliminary experiments using 12-h mycelia showed clear and very strong IM-2-binding activity (2.7 × 10^5 dpm/mg of protein). The dose-response curve of the IM-2-binding activity (Fig. 3a) indicated that IM-2-binding activity increased as protein content increased but deviated from linearity at binding activities greater than 2 × 10^5 dpm/ml. Therefore, the IM-2-binding activity was measured within the linear range. To determine the profile of IM-2-binding activity during growth, mycelia were collected from cultures at 4 to 24 h of cultivation and the specific IM-2-binding activities were measured (Fig. 3b). The activity rapidly increased from 6 h of cultivation, reached a plateau at 10 to 14 h, and decreased sharply there-
after. Because no endogenous IM-2 was produced under the cultivation conditions used and no IM-2 was detected by IM-2 assay (data not shown), competition of [3H]IM-2-C5 with endogenous IM-2 during the binding assay was very unlikely. Therefore, the sharp decrease of the IM-2-binding activity should reflect the degradation or inactivation of the binding protein. The presence of IM-2-binding protein from the early growth phase agrees well with the phenomenon that addition of IM-2 as early as 4 h in the cultivation could induce blue pigment production, suggesting the constitutive nature of the IM-2-binding protein. For further studies, we used the mycelia from 12-h cultures.

**IM-2 binding protein is a cytoplasmic soluble protein.** To check whether DNA or RNA participates in the binding activity, a partially purified preparation was treated with DNase (10 μg/ml, 37°C, 20 min) or RNase (40 μg/ml, 30°C, 30 min), resulting in minor changes in binding activity: a 10 to 30% decrease and an 8% increase after DNase and RNase treatments, respectively. Although the activity decrease induced by DNase treatment was reproducible and beyond experimental error, the actual role of DNase is unknown and needs future investigation. The addition of genomic DNA had no effect on IM-2-binding activity. In good contrast, pronase treatment (0.2 mg/ml, 30°C, 5 min) of crude cell extract or the partially purified preparation completely eliminated IM-2-binding activity, confirming the proteinaceous nature of the IM-2-binding factor.

To ascertain the location of the IM-2-binding protein in the cell, sonicated mycelia were first fractionated by centrifugation at 25,000 × g for 30 min and then the supernatant obtained was successively centrifuged at 100,000 × g for 6 h. All the pellet and supernatant samples were then assayed for binding activity. The IM-2-binding activity was found exclusively in the supernatant fractions (data not shown), indicating that, like the binding proteins for VB (7) and A-factor (11), the IM-2-binding protein is a soluble cytoplasmic protein.

**IM-2-binding protein is specific for IM-2.** The concentration dependence of IM-2-binding activity was studied. The IM-2-specific binding activity in *Streptomyces* sp. strain FRI-5 was found to increase sharply in the nanomolar range, and it reached equilibrium at about 10 nM (Fig. 4a). The Scatchard plot (17) gave a straight line, and the slope of the line indicates a Kd of 1.3 nM (Fig. 4b), which is quite low and almost the same as the Kd of VB-binding protein for VB (1.1 nM) (6). In sharp contrast to the very high affinity toward IM-2, the binding protein of *Streptomyces* sp. strain FRI-5 showed much lower affinity toward [3H]VB-C5 (2-(1'-α-hydroxy-[6',7',8'-3H] heptyl)-3-(hydroxymethyl)butanolide) (Kd = 35 nM) (Fig. 4c), implying that the binding protein of *Streptomyces* sp. strain FRI-5 is highly specific for IM-2.

FIG. 2. Synthetic pathway for [3H]IM-2-C5. Experimental details are described in Materials and Methods. TMS, trimethylsilyl.
Further confirmation of this ligand specificity came from a competitive binding assay, in which several autoregulators, i.e., IM-2-C₄ [2-(1'-β-hydroxybutyl)-3-(hydroxymethyl)butanolide], IM-2-C₅, VB-C₄ [2-(1'-α-hydroxybutyl)-3-(hydroxymethyl)butanolide] VB-C (natural form), A-factor-C₄ [2-(1'-keto-butyryl)-3-(hydroxymethyl)butanolide], and A-factor (natural form), were used as competitive nonlabeled ligands against [³H]IM-2-C₅. The experiments were conducted at 1.5-, 15-, and 150-fold molar excesses of cold ligands. Again, IM-2 type compounds were shown to be the most effective ligands at each concentration, while the VB type was less so and the A-factor type was very poor (Fig. 5). At a 150-fold molar excess, the relative effectiveness of VB-C was 86.3% of that of IM-2-C₅, which agreed well with the calculated value (85%) from the corresponding $K_d$ values (35 nM for VB and 1.3 nM for IM-2). Although all the ligands have the γ-butyrolactone structure in

![Graphs showing IM-2 binding activity vs. protein concentration](image)

**FIG. 3.** (a) Correlation between the amount of crude cell extract and IM-2-binding activity. Binding activity was assayed by equilibrium dialysis with an increasing amount of crude cell extract. (b) Time course of specific IM-2-binding activity during cultivation of *Streptomyces* sp. strain FRI-5. Crude cell extracts were prepared from mycelia after the indicated cultivation times and assayed for IM-2-binding activity by equilibrium dialysis. ■, specific IM-2-binding activity; ○, mycelial wet weight of *Streptomyces* sp. strain FRI-5.

![Graphs showing specific IM-2 binding activity vs. incubation time](image)

**FIG. 4.** (a) Concentration dependence of [³H]IM-2 binding in the absence (●) and presence (▲) of nonlabeled IM-2-C₅. The specific binding (not shown) is the difference between the total and nonspecific binding. (b and c) Scatchard plots of specific [³H]IM-2-C₅ binding (b) and [³H]VB-C₇ binding (c). For measuring [³H]VB-C₇ binding, [³H]IM-2-C₅ and cold VB-C were used.
common, minor differences in the state of oxidation or configuration at C-1' (see structures in Fig. 5) seem to have a drastic effect on their affinity.

An additional finding was that VB-C_4 showed about 100-fold less affinity than did the IM-2 type (comparison between VB-C_4 and the IM-2 type at 1.5- to 150-fold molar excesses) but was still more effective than VB-C containing a C-2 side chain of two more carbons. This finding demonstrates that the length of the C-2 side chain is also important in the affinity.

**Determination of M_r of IM-2-binding protein.** The M_r of the native IM-2-binding protein was estimated by using a partially purified sample by gel filtration HPLC in the presence of 0.1, 0.3, or 0.5 M KCl. An apparent M_r of about 60,000 was observed at all the ionic strengths (Fig. 6). The apparent M_r of the VB-binding protein is affected by the ionic strength, probably by hydrophobic interaction with the gel resin (7); the fact that

![Graph showing molecular weight against retention time](http://jb.asm.org/)

**TABLE 1. Purification of IM-2-binding protein from Streptomyces sp. strain FRI-5 starting with 100 g of wet mycelia**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (10^7 dpm)</th>
<th>Sp act (10^5 dpm/mg protein)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
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<td>100</td>
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<td>0.9</td>
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<td>3.1</td>
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<tr>
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<td>16.8</td>
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<tr>
<td>Phenyl-5PW HPLC</td>
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<td>15.5</td>
<td>831.8</td>
<td>305.7</td>
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</table>

FIG. 5. Effectiveness of several autoregulators as competitive ligands in the binding of [3H]IM-2-C_5 to IM-2-binding protein. Nonlabeled ligands are present at 1.5-fold (dotted bars), 15-fold (hatched bars), and 150-fold (black bars) molar excesses over [3H]IM-2-C_5. The structures of competitive ligands are indicated on the right. "Racemic" indicates a 1:1 mixture of the natural compound and its enantiomer, e.g., racemic IM-2-C_5 contains a natural isomer having (2R,3R,1'R) configuration (12) and its (2S,3S,1'S) enantiomer.

FIG. 6. Molecular weight determination of IM-2-binding protein by gel filtration HPLC. The elution position of the IM-2-binding protein is indicated by an arrow. The following proteins (black circles) were used as standards: glutamate dehydrogenase (M_r, 290,000), lactate dehydrogenase (M_r, 142,000), yeast enolase (M_r, 67,000), yeast adenylate kinase (M_r, 32,000), and cytochrome c (M_r, 12,400). The buffer used was 0.02 M triethanolamine-HCl, pH 7.0, containing 0.3 M KCl and 5 mM dithiothreitol.
FIG. 7. Elution pattern of IM-2-binding protein from hydrophobic (phenyl-5PW) HPLC. (a) Fluorescence. (ex. 280 nm, em. 340 nm). Numbers above each peak correspond to fractions indicated in panels b and c. (b) Specific IM-2-binding activities of fractions. (c) SDS-PAGE analyses of active fractions. A 500-μl volume of each fraction was analyzed on a ready-made 4 to 20% gradient SDS-PAGE gel (see Materials and Methods for details) and stained with Coomassie brilliant blue G-250. The positions of molecular mass makers and fraction numbers are indicated on the left and at the top, respectively.
the IM-2-binding protein is not affected suggests that it is more hydrophilic than the VB-binding protein.

Purification of IM-2-binding protein. Purification of the IM-2-binding protein by using 100 g of wet mycelia as the starting material is summarized in Table 1. Binding activity in the (NH₄)₂SO₄-precipitated fraction was inhibited partly by the coexisting (NH₄)₂SO₄. The IM-2-binding protein was purified about 306-fold with a yield of 4.3%. The final fraction obtained after the phenyl-5PW HPLC step showed significantly greater specific binding activity (fraction 6 in Fig. 7a and b). SDS-PAGE analysis of the active fractions revealed that the band of about 27 kDa is common (Fig. 7c). The most active fraction (fraction 6) contained a minor contaminant of 32 kDa, but the contamination was much more dominant in a less active fraction (fraction 7), which was thus eliminated from the group of possible candidates for the IM-2-binding protein. Therefore, the protein of 27 kDa was concluded to be the IM-2-binding protein of Streptomyces sp. strain FRI-5. This Mᵣ of the IM-2-binding protein matches well those of the VB-binding protein (26,000 [our unpublished data]) and the A-factor-binding protein (26,000 [11]). Considering that the Mᵣ is about 60,000 under nondenaturing conditions, the IM-2-binding protein seems to present as a dimer in the native state.

Following the recent purification of the VB-binding protein (our unpublished data), the IM-2-binding protein is only the second binding protein of this type to be identified and purified almost to homogeneity. Because the amount of this kind of binding protein is very small, cloning of the corresponding gene and finding a way to prepare a recombinant IM-2-binding protein are necessary for its further characterization; partial amino acid sequencing and molecular cloning are at present under way in our laboratory for this purpose. Since corresponding binding proteins have been found for all three classes of butyrolactone autoregulators from Streptomyces species—the IM-2, VB-, and A-factor-binding proteins—binding proteins are most likely participating in autoregulator-dependent differentiation in Streptomyces spp. The availability of purified IM-2- and VB-binding proteins may assist in elucidating the actual mechanism of signal transduction.

REFERENCES


