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# Isatin-induced increase in the affinity of human ferrochelatase and adrenodoxin reductase interaction

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Abstract:: Isatin (indol-2,3-dione) is an endogenous non-peptide regulator exhibiting a wide range of biological and pharmacological activities, which are poorly characterized in terms of their molecular mechanisms. Identification of many isatin-binding proteins in the mammalian brain and liver suggests that isatin may influence their functions. We have hypothesized that besides direct action on particular protein targets, isatin can act as a regulator of protein-protein interactions (PPIs). In this SPR-based biosensor study we have found that physiologically relevant concentrations of isatin (25-100 μM) increase affinity of interactions between human recombinant ferrochelatase (FECH) and NADPH-dependent adrenodoxin reductase (ADR). In the presence of increasing concentrations of isatin the Kd values demonstrated a significant (up to 6-fold) decrease. It is especially important that the interaction of isatin with each individual protein (FECH, ADR) was basically negligible and therefore could not contribute to the observed effect. This effect was specific only for the FECH/ADR complex formation and was not observed for other protein complexes studied: FECH/CYB5A and FECH/SMAD4.

Keywords: ferrochelatase; NADPH-dependent adrenodoxin reductase; human; protein-protein interactions; isatin; surface plasmon resonance; interactomics

1. Isatin a small molecule non-peptide regulator of various protein-protein interactions significantly increased affinity of complex formation between human ferrochelatase (FECH) and NADPH-dependent adrenodoxin reductase (ADR) investigated by means of Surface Plasmon Resonance technology.

2. This effect was specific (and was not observed for other complex formations FECH/CYB5A and FECH/SMAD4.

3. Isatin increased affinity of FECH/ADR complex formation in a concentration dependent manner.

*Abbreviations:* PPI, protein-protein interaction; SPR, surface plasmon resonance; RU, response units; Kd - dissociation constant; FECH, ferrochelatase; ADR, NADPH-dependent adrenodoxin reductase; CYB5A, cytochrome b5 microsomal; SMAD4, member 4 of the SMAD family of signal transduction proteins.

**Introduction**

Isatin is an oxidized indole exhibiting a wide range of biological and pharmacological effects.[1-5] Endogenous isatin has been found in mammalian brains, peripheral organs and body fluids.[6-9] Physiological concentrations of isatin are usually in the micromolar range, but in some tissues may reach 50 - 100 μM. Mechanisms of numerous biological and pharmacological activities of isatin still need better understanding. The latter is especially important in the context of numerous isatin-binding proteins recognized in the mammalian brain and liver.[10-13] We have hypothesized that in addition to direct action on particular protein targets, isatin can act as a regulator of protein-protein interactions (PPIs). Indeed, results of our recent studies suggest that isatin can decrease affinity of some PPI.[14-15] In this study we performed an SPR-based biosensor analysis for PPIs, which would demonstrate altered affinity in the presence of isatin. We found one pair of proteins, human FECH and ADR, that demonstrated a large increase in the affinity of their interaction in the presence of physiological concentrations of this regulator (25 -100 μM). Interaction between these proteins was found earlier by means of direct molecular fishing and subsequent SPR-based validation of this interaction.[16-18]

**Results and discussion**

Using the SPR technology, we have investigated the interaction between FECH, immobilized on the optical chip surface, and ADR in the presence and absence of isatin. Figure 1 shows typical series of sensorgrams obtained at different concentrations of ADR and isatin. In the presence of isatin, the amount of ADR, bound to immobilized FECH markedly increased. Figure 3 shows the

concentration dependence of the isatin effect on the Kd values for the ADR/FECH complex formation. It can be seen that in the range of physiological concentrations of isatin (25-100 μM) the affinity increases between 2 and 6 times, while at a higher isatin concentration (270 μM) this effect becomes even more pronounced (more than 15 times). In order to evaluate the specificity of isatin action on PPIs we have also investigated its effect on the interaction between immobilized FECH and two other proteins (signal transduction protein SMAD4 and CYB5A) (Figure 2A) earlier recognized as FECH-binding proteins.[18] FECH interacted with both CYB5A and SMAD4 (Table and Suppl. Fig.S1-S3), but not with other proteins used as controls (Suppl. Fig.S4). However, isatin had no effect on FECH/CYB5B and FECH/SMAD4 interactions (see Table) thus indicating specificity of its effect on the FECH/ADR interaction. It is especially important that the interaction of isatin with each individual protein (FECH, ADR) was almost negligible and therefore could not contribute to the observed effect (Figure 2B).

Biological role of the isatin effect on the FECH/ADR interaction requires further investigation. FECH and ADR are mitochondrial proteins. The former is the enzyme that catalyzes insertion of iron into the protoporphyrin IX ring with formation of heme *b*, a prosthetic group of many hemoproteins. Certain evidence exists that ADR plays a role in the maturation of both heme and iron–sulfur clusters.[19] It is suggested that this enzyme could serve as "a reducing agent for the iron, a necessary step before its assimilation into protoporphyrin IX by ferrochelatase".[19] In this context our results on physical interaction between FECH and ADR provide an important experimental support for this hypothesis.

Thus, results of this study provide first experimental evidence for an isatin-induced increase in PPIs. This strengthens a recently proposed role of isatin as a regulator of PPIs in living organisms. Taken together with previous data on decreased affinity of some PPIs in the presence of isatin [14-15] these results indicate that isatin can exhibit multidirectional effects on PPIs: an increase in affinity of one type of PPIs and a decrease in affinity of other type of PPIs. At the same time,

insensitivity of some pairs of PPIs investigated in this study to isatin also indicates that the regulatory effects of isatin on PPI are rather specific and do not cover all possible PPIs.

## Materials and methods

### *Recombinant proteins*

Highly purified preparations (> 95% by SDS-PAGE) of human NADPH-dependent adrenodoxin reductase (ADR) and cytochrome b5 (CYB5A) were expressed and purified in the Institute of Bioorganic Chemistry NAS (Belarus Republic). Methods of their expression in *E. coli* cells as hexahistidine tagged proteins, isolation, purification, and functional analysis have been described earlier.[20-22] SMAD and FECH were expressed in bacteria as His-tagged proteins and purified to homogeneity by means of metal-affinity chromatography followed by chromatography on hydroxyapatite and dialysis.

### *Surface plasmon resonance (SPR) analysis*

SPR analysis was carried out using optical biosensor Biacore 3000 (GE Healthcare, USA) at 25°C and sensor chips CM5. Running buffer HBS-EP+ (150 mM NaCl, 1 mM EDTA, 0.05% Tween-20, 2 mM dithiothreitol, 10 mM HEPES, pH 7.5) with 2 mM dithiothreitol was used at a flow rate of 5 μL/min. FECH was employed as a ligand, immobilized on the chip surface using standard covalent amino coupling protocol described in the Sensor surface handbook (GE Healthcare, USA) in 10 mM sodium acetate (pH 4.0). The flow rate used for all immobilization steps was 5 μL/min. The chip surface was activated by injection of a 1:1 mixture of 50 μL EDC and NHS (25 μL 400 mM EDC and 25 μL 100 mM NHS) for 7 min. The FECH solution in immobilization buffer (0.05 mg/ml protein in sodium acetate buffer) was then injected into the activated flow cell. Unreacted groups on the CM5 chip surface were capped with 1M ethanolamine-HCl (pH 8,5). The signal biosensor change corresponding to immobilization of FECH was about 6000 response units (RU), 1 RU are

equal to 1 pg material bound on 1 $mm^2$ chip surface. In the control flow cell the reaction was carried using the same protocol, but without protein immobilization step. Samples of CYB5A, SMAD4 and ADR dissolved in the running buffer were injected as analytes for 10 min at a flow rate of 10 μL/min. Solution containing 5 mM NaOH was used for the regeneration of the chip surface for 15 s at a flow rate of 25 μL/min. The control line data (the signal from the channel with carboxymethyl dextran without the immobilized FECH) was subtracted from the raw data, obtained from the flow cell with the immobilized FECH. The $k_{on}$ and $k_{off}$ values were calculated in the BIAevaluation 4.1 software from the sensorgrams using the Langmuir binding model (1:1 complex formation) and $K_d$ values were calculated as ratio: $K_d = k_{off}/k_{on}$.

***Evaluation of the isatin effect on protein-protein interaction (PPI)***

The effect of isatin on affinity of protein complex formation was evaluated by comparison of sensorgrams obtained in the presence or the absence of isatin. Protein samples (0.5 μM – 10 μM) in running buffer were mixed with isatin ethanol solution up to final concentrations of isatin: 25 μM, 50 μM, 100 μM and 270 μM. The final concentration of ethanol was less than 1% (v/v). After incubation of protein samples for 15 min at +4°C they were injected through the biosensor channel with immobilized FECH by using Quickinject mode for 10 min at a flow rate of 10 μL/min. In control experiments, the protein samples were incubated in the HBS-EP+ buffer (1% v/v ethanol) without isatin.

**Supplementary material**

Supplementary material contains additional SPR data.

**Acknowledgement**

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**Table 1.** The effect of isatin on ADR, CYB5A and SMAD4 interaction with immobilized FECH.

| PPI | Isatin | | | | | |
|---|---|---|---|---|---|---|
| | 0 μM | | | 100 μM | | |
| | $k_{on}$ ($M^{-1}$ $s^{-1}$) | $k_{off}$ ($s^{-1}$) | $K_d$ (μM) | $k_{on}$ ($M^{-1}$ $s^{-1}$) | $k_{off}$ ($s^{-1}$) | $K_d$ (μM) |
| ADR/FECH | $4.2\pm0.4\cdot10^{2}$ | $5.6\pm0.2\cdot10^{-3}$ | 13.0 ± 2.0 | $1.5\pm0.2\cdot10^{3}$ | $3.1\pm0.1\cdot10^{-3}$ | 2.0 ± 0.5 |
| CYB5A/FECH | $3.3\pm0.3\cdot10^{2}$ | $5.2\pm0.2\cdot10^{-4}$ | 1.6 ± 0.5 | $3.5\pm0.3\cdot10^{2}$ | $5.2\pm0.2\cdot10^{-4}$ | 1.5 ± 0.4 |
| SMAD4/FECH | $3.1\pm0.3\cdot10^{2}$ | $4.5\pm 0.2\cdot10^{-4}$ | 1.5 ± 0.7 | $2.9\pm0.3\cdot10^{2}$ | $5.0\pm0.2\cdot10^{-4}$ | 1.7 ± 0.5 |

Data represent mean ± SEM for a series of repeated measurements (n = 3).

**Figure 1.** . The effect of isatin on interaction of ADR with immobilized FECH.

**Figure 2.** Binding of protein partners (ADR, CYB5A, SMAD4, final concentration 5 μM) with immobilized FECH in the presence or in the absence of 270 μM isatin (A), and binding of 100 μM or 270 μM isatin with individual proteins, FECH and ADR (B). Data representing mean values (± SD) of at least three independent experiments demonstrate low isatin binding to individual proteins.

**Figure 3.** Dependence of Kd of FECH/ADR protein complex on isatin concentration.

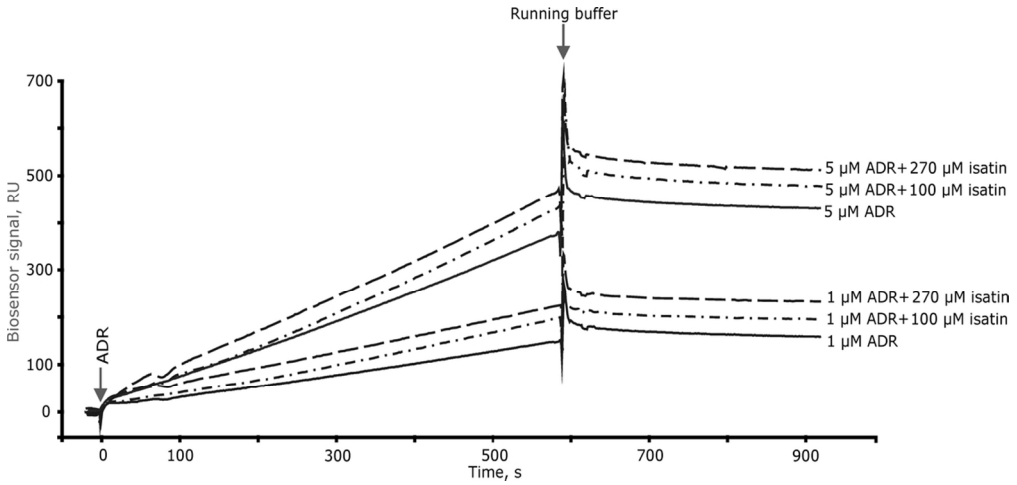


Figure 1. The effect of isatin on interaction of ADR with immobilized FECH.

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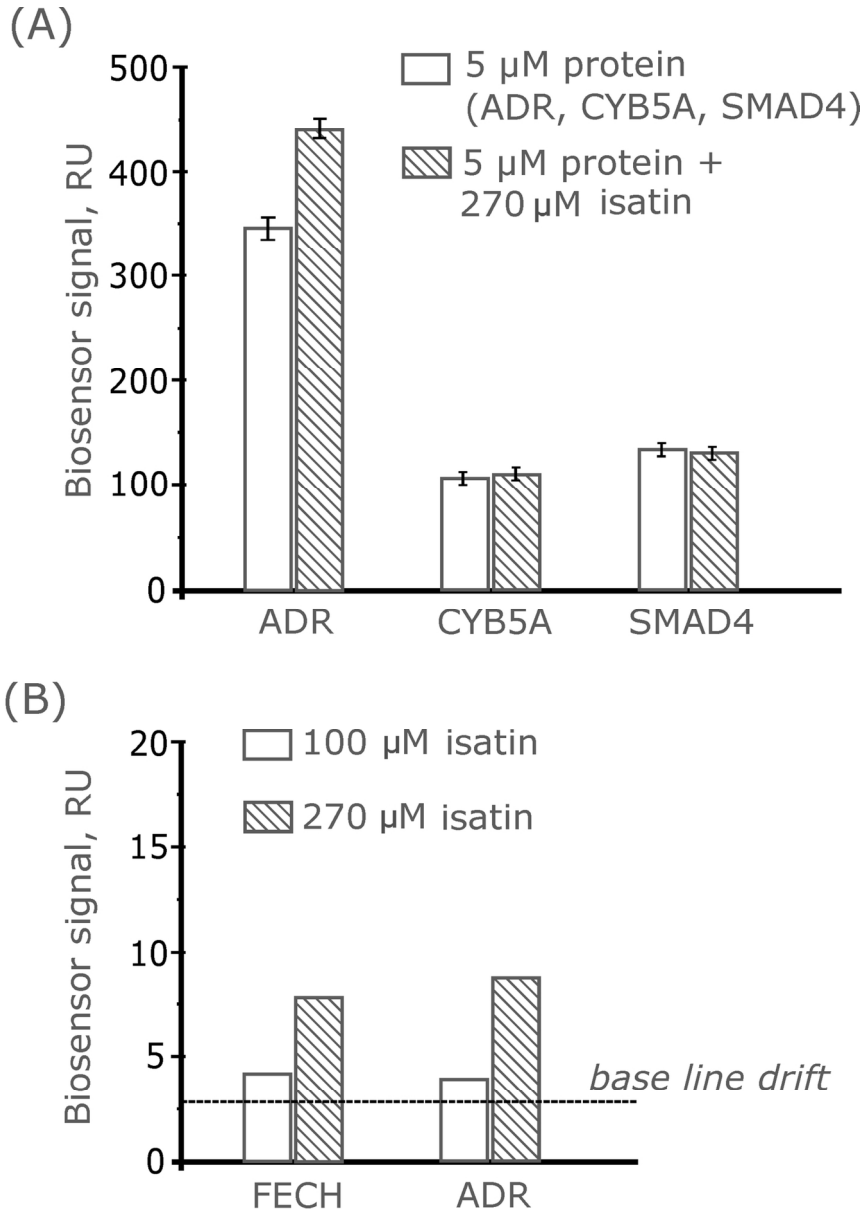


Figure 2. Binding of protein partners (ADR, CYB5A, SMAD4, final concentration 5 μM) with immobilized FECH in the presence or in the absence of 270 μM isatin (A), and binding of 100 μM or 270 μM isatin with individual proteins, FECH and ADR (B). Data representing mean values (± SD) of at least three independent experiments demonstrate low isatin binding to individual proteins.

121x170mm (300 x 300 DPI)

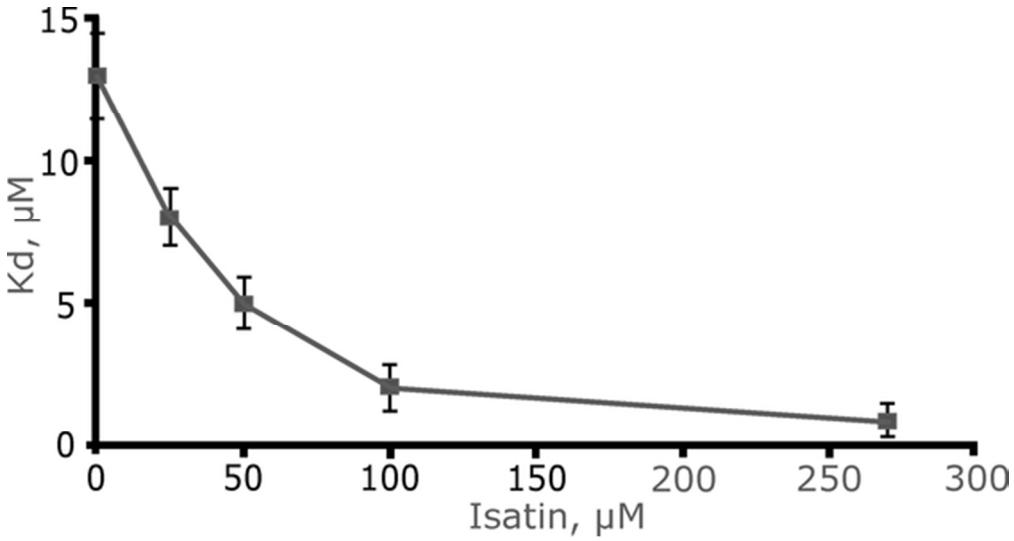


Figure 3. Dependence of Kd of FECH/ADR protein complex on isatin concentration.

53x28mm (300 x 300 DPI)