RESEARCH ARTICLE

Isatin-binding proteins of rat and mouse brain: Proteomic identification and optical biosensor validation

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Isatin (indole-2,3-dione) is an endogenous indole that has a distinct and discontinuous distribution in the brain and in other mammalian tissues and body fluids. Its output is increased under conditions of stress and anxiety. Isatin itself and its analogues exhibit a wide range of pharmacological activities but its specific biological targets still are not well characterized. Affinity chromatography of Triton X-100 lysates of soluble and particulate fractions of mouse and rat whole brain homogenates on 5-aminocaproyl-isatin-Sepharose followed by subsequent proteomic analysis resulted in identification of 65 and 64 individual proteins, respectively. Isatin-binding capacity of some of the identified proteins has been validated in an optical biosensor study using a Biacore 3000 optical biosensor, 5-aminocarproyl-isatin, and 5-aminoisatin as the affinity ligands. The K_d values (of 0.1–20 μM) obtained during the optical biosensor experiments were consistent with the range of K_d values recently reported for [^{3}H]isatin binding to brain sections. Although the number of isatin-binding proteins identified in the mouse and rat brain was similar, only 21 proteins (about one-third) were identical in the two species. This may be one reason for the differences in isatin effects in rats and mice reported in the literature.

Keywords:
Animal proteomics / Brain / Isatin / Optical biosensor

1 Introduction

Isatin is an endogenous indole that is widely distributed in mammalian brain, peripheral tissues, and body fluids (for reviews see [1–3]). The isatin moiety is also present in a range of compounds, which can act as inhibitors of apoptosis, anticonvulsants, anxyolytics, and other antiviral, anti-bacterial, antitubercular, anti-fungal, and anticancer agents (for reviews see [3–5]). It is possible that drugs containing the isatin moiety may compete with endogenous isatin, and the latter can influence their therapeutic effect [3]. Consequently, analysis of the isatin content of the body, its metabolic routes, and its interactions may not only be of importance for understanding more about the function of this endogenous regulator, but also for understanding its possible role as a functional agonist/antagonist of drugs. Physiological concentrations in blood can exceed 1 μM [6, 7]. Basal tissue concentrations are generally in the range of 0.1–1 μM [2]; however, in some tissues much higher levels (40–70 μM) may also occur [3]. Acute stress in rats increased isatin level in the brain [7], plasma [7], and urine [7, 8].

Isatin itself has a wide spectrum of behavioural and metabolic effects that have been studied in rats and mice [1–3, 9, 10]. *In vitro* physiological concentrations of isatin (0.01–0.4 μM) antagonize atrial natriuretic peptide receptor binding and nitric oxide-stimulated guanylyl cyclase [11–13]. Higher (micromolar) concentrations inhibit monoamine oxidase B [1] and the complex of isatin with pure monoamine oxidase B has been crystallized [14].

[^{3}H]isatin imaging *in situ* has revealed the existence of isatin-binding sites in various structures of rat brain [15].

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Abbreviation: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

Binding of [^{3}H]isatin to rat brain sections was saturable and characterized by K_d values (of 0.2–0.3 μM), consistent with physiological concentrations [16]. However, in most brain regions the homologous inhibition of [^{3}H]isatin binding by increasing concentrations of cold isatin demonstrated complex behaviour, suggesting involvement of various binding proteins characterized by different affinity to isatin [16]. Indeed, affinity chromatography of Triton X-100 lysates of whole brain homogenates on 5-aminocaproyl-isatin-Sepharose followed by subsequent proteomic analysis resulted in identification of 25 individual proteins [16]. One of them, glyceraldehyde-3-phosphate dehydrogenase, a glycolytic enzyme exhibiting some other important functions (*e.g.* regulation of apoptosis) has earlier been known as one of few isatin-binding proteins [17]. However, several expected and well-documented targets of isatin were not found. It also remains unclear whether proteomic profiles of rat and mouse isatin-binding proteins are the same and whether other identified proteins would demonstrate isatin binding as purified glyceraldehyde-3-phosphate dehydrogenase.

Thus, in the present study we have compared the proteomic profiles of isatin-binding proteins in rat and mouse brain and validated the isatin-binding capacity of some of them using a Biacore 3000 optical biosensor and 5-aminocarproyl-isatin and 5-aminoisatin as the affinity ligands for the identified isatin-binding proteins.

2 Materials and methods

2.1 Chemicals

Cyanogen bromide-activated-Sepharose 4B, DTT, iodoacetamide, trypsin, Tris (hydroxymethyl) aminomethane, guanidine hydrochloride, ammonium hydrocarbonate, sodium chloride, Triton X-100, ACN, formic acid, CBB G-250 were purchased from Sigma-Aldrich (USA).

Acetic acid (sodium salt), boric acid, sodium tetraborate, sodium hydroxide were from Acros Organics (USA).

HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20); amine coupling kit, 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride, *N*-hydroxysuccinimide; ethanolamine-HCl; research grade sensor CM5 chips carrying hydrophilic carboxymethylated dextran matrix were purchased from Biacore (GE, USA).

5-aminoisatin was synthesized as described earlier [18].

2.2 Animals and preparation of brain fractions

Male Wistar rats (250–300 g) and male C57BL/6 mice (20–25 g) obtained from the Stolbovaya nursery (Russian Academy of Medical Sciences) were used in the experiments that were performed at least 1 wk after their arrival from the nursery. Animals received a standard laboratory chow and water *ad libitum* and their decapitation was performed between 11.00–13.00. The brains were immediately dissected and homogenized in 0.05 M potassium-phosphate buffer, pH 7.4, using Ultra-Turrax T 10 homogenizer at a low speed, to obtain 30% w/v homogenate.

2.3 Preparations of purified proteins

Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was purified as described previously [17]. Recombinant human peroxiredoxin 1 expressed in *Escherichia coli*, rabbit muscle glycogen phosphorylase, and creatine phosphokinase were obtained from Sigma-Aldrich.

2.4 Preparation of 5-aminocaproyl-isatin-Sepharose

N-(6-aminocaproyl)-5-aminoisatin synthesized as described earlier [16] was coupled to cyanogen bromide-activated Sepharose 4B according to standard protocols. The amount of immobilized isatin analogue (about 0.24 μmol/mL of suspension (1:1 v/v)) was evaluated by determining the number of reactive groups of cyanogen bromide-activated Sepharose [16].

For evaluation of non-specific binding of proteins onto the affinity sorbent we also prepared control cyanogen bromide-activated Sepharose, which was subjected to the same treatments as 5-aminocaproyl-isatin-Sepharose but without 5-aminocaproyl-isatin.

2.5 Isolation and identification of isatin-binding proteins

The whole brain rat or mouse homogenate (protein concentration about 40 mg/mL) prepared using 0.05 M potassium-phosphate buffer, pH 7.4, was initially centrifuged at 500 × *g* for 5 min to remove debris. Particulate and soluble fractions obtained by subsequent centrifugation at 16 000 × *g* for 60 min were then separately treated with Triton X-100. The particulate fraction was resuspended (protein concentration 30 mg/mL) in 0.05 M potassium-phosphate buffer, pH 7.4, and lysed with 3% Triton X-100 (final concentration) for 1 h in the cold (+4°C). Then the preparation was diluted three times with the same buffer and centrifuged at 16 000 × *g* for 30 min. Supernatant (protein concentration 30 mg/mL) was incubated with 3% Triton
X-100 and centrifuged as above.

5-aminocaproyl-isatin-Sepharose was loaded with cleared lysates of soluble or particulate fractions separately (about 10 mg/mL). Affinity coupling was performed for 2 h in a suspension (1:1) at 4°C under gentle stirring. The affinity

sorbent was washed with 100 volumes of the buffer, and the amount of protein on Sepharose was determined by modified Bradford method using Sepharose with the immobilized isatin derivative as a reference [19]. Affinity elution was performed with the column (1 × 2 cm) at room temperature and a flow rate 0.5 mL/min with 1 mM isatin in 0.05 M potassium-phosphate buffer, pH 7.4 (30 mL), and then with 1 M NaCl in the same buffer (30 mL).

In parallel, we evaluated non-specific binding of brain lysates using a control batch of cyanogen bromide-activated Sepharose prepared exactly as the affinity sorbent (including sorbent deactivation) but without addition of the affinity ligand.

Eluate obtained (30 mL) was concentrated up to 0.25 mL using Amicon Ultra centrifugal filter devices (Millipore, USA). Proteins were extracted using chloroform-methanol mixture [20], modified by reduction with 0.02 M DTT in 6 M guanidine chloride, pH 6.8, (1 h, 37°C), and subsequent carboxymethylation of SH groups with 0.17 M iodoacetamide (1 h, 37°C). After the same extraction procedure [20] the protein sediment was dissolved in 50 mM ammonium hydrocarbonate and sonicated in the sonication bath for 15 min at 4°C. After trypsinolysis (1 μg trypsin/100 μg of sample protein, 37°C, 1 h; then 2 μg trypsin/100 μg of sample protein, 37°C, overnight) formic acid was added to the preparations up to 0.5%. The preparations were evaporated using a vacuum concentrator 5301 (Eppendorf, AG, Germany), then dissolved in 0.1% formic acid, and analyzed by LC-MS/MS. Reverse-phase nano-LCMS/MS was performed using an Agilent 1100 nano-flow HPLC-Chip cube system coupled to Agilent 1100 XCT Ultra Series MSD Trap (Agilent Technologies, Palo Alto, CA, USA). Tryptic peptides were separated on the HPLC Chip (40 nL trap column, 75 μm × 43 mm analytical column, 5 μm C-18SB-ZX, Agilent Technologies) using a linear gradient of 5–80% ACN in 0.1% formic acid over 60 min at a flow rate of 300 nL/min and detected by an ion trap in 300–1800 m/z range following the supplier's recommendations. Mass spectra were acquired in positive-ion mode with automated data-dependent MS/MS on the five most intense ions from precursor MS scans.

Protein identification was performed using Spectrum Mill MS Proteomics Workbench Rev A.03.03.078 (Agilent Technologies). Protein identifications were obtained by comparison of experimental data to the Swiss-Prot rat and mouse subset database. The following search parameters were used: trypsin was used as the cutting enzyme, mass tolerance for the monoisotopic peptide window was set to ±1.5 Da, the MS/MS tolerance window was set to ±0.5 Da, and two missed cleavage were allowed. Cysteine carbamidomethylation was chosen as a fixed modification and oxidized methionine was chosen as a variable modifications. The criteria of positive identification were set as follows: 70% minimum scored peak intensity, δ-forward-reverse score >2; at least two peptides identifications with a confident score >7 and summarized protein score >13 [21].

2.6 Optical biosensor measurements

2.6.1 Covalent immobilization of ligands on the CM5 sensor chip surface

Measurements were performed with a Biacore 3000 instrument (GE), thermostated at 25°C. The surface of CM5 chip was activated following a standard 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride/*N*-hydroxysuccinimide amine coupling Biacore protocol. The 31 mM 5-aminoisatin or 36 mM 5-aminocaproyl-isatin in 20 mM borate buffer, pH 8.5, with 28% DMSO was injected over an activated chip surface for 7 min at 5 μL/min followed by 5 min injection of HBS-EP buffer to remove excess ligand, and 1 min injection of 1 M ethanolamine, pH 8.5, to inactivate residual active groups.

2.6.2 Binding measurements

Ligand–protein interactions were monitored by injecting proteins dissolved at various concentrations in 50 mM KP buffer (running buffer) at the flow rate of 5 μL/min for 5 min. The sensor surface was regenerated between sample injections by washing with 6 M guanidine-HCl for 0.5 min at the flow rate of 50 μL/min. Interactions were estimated by subtracting the response in a blank flow cell from the response in a cell with immobilized ligands.

2.6.3 Data processing

2.6.3.1 Steady-state analysis of SPR data

Data analysis was done by applying the BIAevaluation v.4.1 software. To determine the equilibrium dissociation constant ($K_d = 1/K_A$) for the interaction, the equilibrium data were fitted to a single-site model (Eq. 1),

$$R_{eq} = (R_{max} \cdot C \cdot K_A)/(1 + C \cdot K_A) \quad (1)$$

where R_{max} stands for the maximal response; C is the concentration of a protein; R_{eq} is the equilibrium response at given protein concentration; and K_A is the equilibrium association constant.

2.6.3.2 Kinetic analysis of SPR data

Kinetic rate constants were calculated from the sensorgrams (Figs. 1 and 2) by globally fitting response curves obtained at various ligand concentrations to the 1:1 binding model with a drifting baseline. Association (k_{on}) and dissociation (k_{off}) rate constants were fitted simultaneously using Eq. 2:

$$dR/dt = k_{on} \cdot C \cdot (R_{max} - R) - k_{off} \cdot R \quad (2)$$

where, R stands for the biosensor response of the formed complex; C is the concentration of the protein; R_{max} is the maximal theoretical value of binding response for a given protein.

Table 1. Proteomic identification of rat brain isatin-binding proteins

No.	Protein name	Database accession number	MW, Da	p*I*	No. of peptides	Sequence coverage, %	Mill search score
I Proteins/enzymes involved in energy generation and carbohydrate metabolism ($n = 15$)							
1	Pyruvate dehydrogenase (lipoamide) β	gi:50925725	38 982.4	6.21	3	13	145.60
2	Glycogen phosphorylase (EC 2.4.1.1), brain	gi:480806	96 742.5	6.24	7	10	69.97
3	Ckb protein	gi:56388799	45 248.5	5.40	3	9	57.21
4	Phosphofructokinase, muscle	gi:13929002	85 560.4	8.23	4	7	53.99
5	Triosephosphate isomerase (TIM)	gi:1351280	26 921.1	6.45	4	24	51.27
6	Glutamate synthetase 1	gi:76496512	46 486.7	6.71	3	9	44.53
7	ATPase, H+ transporting, V1 subunit A, isoform 1	gi:34869154	68 265.4	5.42	3	8	41.92
8	Eno1 protein	gi:38649320	51 255.8	6.69	3	9	41.37
9	Citrate synthase	gi:18543177	51 867.1	8.53	2	6	30.07
10	Aconitase 2	gi:38541404	85 433.9	7.87	2	6	28.83
11	Pyruvate dehydrogenase E1 α form 1 subunit	gi:34879653	43 275.1	8.49	2	5	28.81
12	Glyceraldehyde-3-phosphate dehydrogenase	gi:62658024	36 015.4	8.14	2	8	27.14
13	Malate dehydrogenase 2, NAD (mitochondrial)	gi:38648863	35 683.8	8.93	2	8	26.99
14	Dihydrolipoamide dehydrogenase (E3 component of pyruvate dehydrogenase, 2-oxo-glutarate dehydrogenase, branched chain keto acid dehydrogenase complexe)	gi:38303871	54 038.4	7.96	2	7	24.85
15	ATPase, Na+/K+ transporting, α 2 polypeptide	gi:6978545	112 218	5.39	2	2	23.56 pt
II Proteins involved in cytoskeleton formation, exocytosis, and membrane transport ($n = 19$)							
1	Spna2 protein (αII spectrin)	gi:62644670	284 638.9	5.20	34	19	485.12
2	Adaptor protein complex AP-2, α 2 subunit	gi:51859448	104 174.2	6.40	2	4	146.00
3	Type II keratin Kb1	gi:71051822	59 249.4	8.06	10	16	137.80
4	Microtubule-associated protein 1A	gi:13591886	299 532	4.87	8	4	106.07
5	Actin β	gi:62664168	304 875.1	8.65	6	3	82.13
6	D100 (dynamin)	gi:56054	95 928	6.32	6	9	79.87
7	Valosin-containing protein	gi:38014694	89 349.3	5.14	7	13	79.51
8	Unc-18 protein homolog, 67K	gi:631898	67 520.2	7.98	4	10	75.63
9	Tubulin β chain 15	gi:62663465	101 608.3	5.60	5	10	72.75
10	Non-erythroid spectrin β	gi:33303722	273 587.9	5.50	6	3	72.04
11	Glial fibrillary acidic protein	gi:57032786	49 957.3	5.35	5	12	69.51
12	Tubulin α 1	gi:62653035	50 280	4.94	5	17	66.35
13	Clathrin, heavy polypeptide (Hc)	gi:9506497	191 599.7	5.50	5	4	66.16
14	Tubulin α-2 chain (-tubulin 2)	gi:62653035	50 280.0	4.94	6	19	55.70
15	Type II keratin Kb1	gi:57012354	64 831.2	8.00	4	7	49.24
16	Type II keratin Kb18	gi:62653090	63 712.8	6.32	2	3	37.51
17	Capping protein (actin filament) muscle Z-line, α 2	gi:74355722	32 967.2	5.57	3	15	36.80
18	S100b protein	gi:56270331	10 744.1	4.52	2	40	34.99
19	Adaptor protein complex AP-1, β 1 subunit	gi:8392872	104 589.2	5.00	2	4	31.91
III Proteins/enzymes involved in regulation of gene expression, cell division, and differentiation ($n = 17$)							
1	TOAD-64	gi:599966	62 277.9	5.95	8	24	112.16
2	TBP-interacting protein 120A	gi:16758920	136 632.5	5.52	9	11	121.68
3	Sirtuin (silent mating type information regulation 2 homolog) 2	gi:56605812	39 319.5	6.67	6	24	68.85
4	Nucleolin	gi:55250726	77 276.8	4.66	5	10	64.31
5	Karyopherin (importin) β 1	gi:8393610	97 124.7	4.66	4	6	51.66
6	Heterogeneous nuclear ribonucleoprotein R	gi:62654117	69 619.9	8.57	4	8	46.94
7	Ribosomal protein S3, cytosolic	gi:70850	26 660.4	8.23	4	19	45.96
8	Ac2-067	gi:34876714	24 675.8	4.55	3	28	43.88
9	Heterogeneous nuclear ribonucleoprotein A1 (Hnrpa1)	gi:62718633	24 925.8	7.13	2	10	41.08
10	Heterogeneous nuclear ribonucleoprotein D	gi:13242324	38 192.1	7.61	3	9	38.68
11	Eukaryotic translation elongation factor 1 α 2	gi:15805031	50 150.1	9.10	3	10	36.91
12	Hnrpa3 protein	gi:62659231	26 902.0	7.62	3	15	36.85
13	Hnrpa3 protein	gi:62644588	38 333.9	5.69	12	8	35.46
14	Heterogeneous RNP type A/B HnRNP p40	gi:6562845	36 233	6.48	2	9	31.91
15	Heterogeneous nuclear ribonucleoprotein H2	gi:50927747	49 293.9	5.89	2	8	25.06
16	Ribosomal protein S7	gi:62650074	23 538.6	10.08	2	16	23.86
17	Lysyl-tRNA synthetase	gi:53733461	71 623.6	6.16	2	3	20.81

Table 1. Continued

No.	Protein name	Database accession number	MW, Da	p*I*	No. of peptides	Sequence coverage, %	Mill search score
IV Proteins/enzymes involved in cell signaling and signal transduction ($n = 7$)							
1	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, ζ polypeptide	gi:62990183	27 771.3	4.73	5	20	55.7
2	Cyclic nucleotide phosphodiesterase 1	gi:57977323	47 268.6	9.03	3	9	53.16
3	2′,3′-Cyclic nucleotide 3-phosphodiesterase	gi:203493	45 595.9	9.42	3	13	41.07
4	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, γ polypeptide	gi:9507245	28 302.7	4.80	3	21	40.47
5	G protein β 1 subunit	gi:984553	37 393.2	5.47	3	14	34.00
6	Calmodulin 1	gi:14010863	16 837.7	4.09	2	10	25.35
7	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	gi:13928824	29 121.9	4.55	2	11	22.99
V Antioxidant enzymes and protective proteins ($n = 6$)							
1	Hsc70-ps1	gi: 56385	70 928.5	5.43	11	25	155.88
2	Heat shock protein 1, α	gi:28467005	84 815.3	4.93	2	3	22.46
3	Chaperonin containing TCP1, subunit 2 (β)	gi:54400730	57 458.7	6.01	2	5	22.37
4	Chaperonin subunit 6a (ζ)	gi:75773247	58 017.6	6.63	2	6	20.15
5	84 kDa heat shock protein	gi:51859516	83 341.8	4.97	2	3	19.41
6	Peroxiredoxin 2	gi:34849738	21 797.8	5.34	2	14	17.05

Each protein listed in Tables 1 and 3 was identified at least in three independent experiments, each of which employed independent brain samples as well as their chromatographic and proteomic processing. The cut-off value for the Spectrum MillMS peptide mode was set at 13 [21].

To compensate for a baseline drift, total biosensor response (R_{total}) for a complex is described by Eq. 3:

$$R_{total} = R + R_I + D \cdot (t - t_{on}) \quad (3)$$

where, R_I is a bulk refractive index contribution; D is a slope of the baseline drift; and $t$on is the starting time for sample injection. Equilibrium dissociation constants (K_d) were calculated as a ratio of the rate constants k_{off}/k_{on}.

3 Results

In accordance with our previous study [16] about 4% of proteins from both rat and mouse brain homogenate preparations were specifically adsorbed on the affinity sorbent, 5-aminocaproyl-isatin-Sepharose, whereas the relative content of proteins non-specifically bound to the control Sepharose sorbent was consistently less than 1% of the total protein applied.

Separate loading of cleared Triton X-100 lysates of particulate and soluble fractions of the whole rat brain homogenate onto the affinity sorbent followed by subsequent elution of adsorbed proteins by isatin and NaCl and their proteomic analysis resulted in identification of 64 individual isatin-binding proteins specifically bound to the affinity sorbent (Table 1). Similar experimentation with particulate and soluble fractions of the mouse brain homogenate resulted in identification of 65 individual proteins (Table 3).

Among proteins non-specifically bound to both control Sepharose sorbent and 5-aminocaproyl-isatin-Sepharose we detected only proteins (Tables 2 and 4) that are highly abundant in mouse and rat brain [22].

In order to validate isatin-binding capacity of individual proteins identified in this study we have investigated several available purified proteins. These included rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, creatine phosphokinase, glycogen phosphorylase, and also human peroxiredoxin 1. These proteins exhibit reasonably high interspecies homology (human, rabbit, rat, mice) with corresponding brain proteins identified in this study (glyceraldehyde-3-phosphate dehydrogenase, creatine phosphokinase, glycogen phosphorylase, and also peroxiredoxin 2) [23–27].

Injection of solutions of individual proteins in the running buffer into a carboxymethylated dextran chip containing covalently immobilized 5-aminocaproyl-isatin resulted in the appearance of a biosensor characteristic response (Fig. 1). Its magnitude depended on the amount of the protein added. Subsequent injection of the running buffer caused rapid dissociation of the molecular complexes of glyceraldehyde-3-phosphate dehydrogenase and glycogen phosphorylase, whereas in the case of creatine phosphokinase and peroxiredoxin 1 their dissociation occurred slower. This suggests different affinity of the proteins tested towards 5-aminocaproyl-isatin covalently immobilized on the CM5 chip. Indeed, the K_d values calculated as the k_{off}/k_{on} ratio varied in the range of concentrations 10^{-8}–10^{-5} M; these are consistent with the range of isatin concentrations in various brain structures [1–3]. Since optical biosensor studies of isatin binding to individual proteins originally employed another isatin analogue, 5-aminoisatin, as the affinity ligand

Table 2. Proteomic identification of rat brain proteins non-specifically bound to Sepharose with and without immobilized 5-aminocaproyl-isatin

No.	Protein name	Database accession number	MW, Da	p*I*	No. of peptides	Sequence coverage, %	Mill search score
1	ATP synthase subunit β, mitochondrial precursor	gi 114562	56353.8	5.18	15	47	231.0
2	Keratin, type II cytoskeletal 1	gi 81891716	64831.2	8.00	7	9	99.68
3	Keratin, type I cytoskeletal 10	gi 81891690	56505.3	5.10	6	10	82.14
4	Keratin, type II cytoskeletal 6a	gi 122603096	59249.4	8.06	3	5	52.30
5	Keratin, type II cytoskeletal 5	gi 81170669	61826.0	7.61	3	5	45.16
6	Keratin, type II cytoskeletal 75	gi 166218811	59027.0	8.16	2	4	34.44
7	Keratin, type II cytoskeletal 73	gi 81891700	60387.3	8.17	2	4	32.73
8	Keratin, type I cytoskeletal 42	gi 81891673	50213.4	5.09	2	3	28.19
9	Keratin, type II cytoskeletal 1b	gi 66773802	67254.9	5.48	2	4	24.91
10	Keratin, type I cytoskeletal 17	gi 81891674	48123.0	4.97	1	2	16.42
11	Keratin, type I cytoskeletal 15	gi 81891677	48870.7	4.80	1	2	16.42
12	Keratin, type I cytoskeletal 14	gi 81170667	52684.0	5.08	1	1	16.42
13	Keratin, type I cytoskeletal 12	gi 81891689	48820.4	4.71	1	1	16.42
14	Keratin, type I cytoskeletal 19	gi 73920214	44636.2	5.21	1	2	16.42
15	AP-2 complex subunit β-1	gi 51702208	104553.2	5.22	1	2	19.27

[17], we have also investigated the binding of four individual proteins to the carboxymethylated dextran chip with immobilized 5-aminoisatin (Fig. 2). Results of these binding experiments clearly indicate that all the proteins tested did specifically bind to the carboxymethylated dextran chip with immobilized 5-aminoisatin. However, the calculated K_d values differed from those obtained in the binding experiments with 5-aminocaproyl-isatin as the affinity ligand for the optical biosensor Biacore 3000 (Table 5).

4 Discussion

The present work has provided further insight into isolation and characterization of isatin-binding proteins not only in the rat brain but also in the mouse brain. The first proteomic profiling of rat brain isatin-binding proteins resulted in identification of 25 proteins [16]. In this study we have modified the isolation procedure by separate processing of particulate and soluble fractions of rat and mouse brain homogenates up to the stage of sample trypsinolysis and MS. This resulted in more than 2.5-fold increase in the number of individual proteins identified in the rat brain. It should be noted that previously identified rat brain proteins have been also detected in the present study. This indicates that the isolation procedure is reproducible and suggests that it has been really improved.

Proteomic profiling of isatin-binding proteins from the mouse brain has been performed for the first time. Comparison of the rat and mouse proteomes has shown that the number of individual isatin-binding proteins is basically the same in rat and mouse brains (64 *versus* 65 proteins). The proteins can be subdivided into the groups (Tables 1 and 3): (i) proteins/enzymes involved into energy generation and carbohydrate metabolism; (ii) proteins involved into cytoskeleton formation and exocytosis; (iii) proteins/enzymes involved in regulation of gene expression, protein folding, and cell differentiation; (iv) proteins/enzymes involved in cell signaling; (v) antioxidant enzyme(s). However, the mouse brain isatin-binding proteins also contain an additional group (vi) that is absent in rat brain isatin-binding proteins: proteins/enzymes involved into metabolism of nucleotides and amino acids and related processes. Although qualitative subdivision of isatin-binding proteins is roughly the same, only rat and mouse isatin-binding proteins from the two first groups demonstrated reasonable coincidence varying from 37 to 62% (Table 6). In the other cases this parameter was about 20% or below. However, the isatin-binding proteins identical for mouse and rat brain exhibit undergo similar (although regioselective) changes in brain pathologies (Table 7 [28–59]). Besides altered expression, these include oxidative modification, larger molecular weight associate formation, binding to various proteins, including those associated with the development of particular pathologies. Apart from the group of isatin-binding proteins/enzymes involved in the metabolism of nucleotides and amino acids and related processes, which is absent in rat brain mouse, the highest diversity also includes (iv) proteins/enzymes involved in cell signaling and antioxidant enzyme(s) (v). It is possible that these differences may account for known facts of poor reproducibility of symptoms of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinsonism in rats compared with mice [60] as well as different sensitivity of these species to some other treatments including prolonged immobilization stress [61] or some chemical treatments [62].

Table 3. Proteomic identification of mouse brain isatin-binding proteins

No.	Protein name	Database accession number	MW, Da	p*I*	No. of peptides	Sequence coverage, %	Mill search score
I Proteins/enzymes involved in energy generation and carbohydrate metabolism (*n* = 19)							
1	Aconitate hydratase, mitochondrial precursor (EC 4.2.1.3) (citrate hydrolyase) (Aconitase)	Q99K10	85 464.0	8.08	6	11	89.15
2	Creatine kinase B-type (EC 2.7.3.2) (creatine kinase, B chain) (B-CK)	Q0447	42 713.5	5.40	6	18	86.47
3	Pyruvate dehydrogenase E1 component subunit β, mitochondrial precursor (EC 1.2.4.1) (PDHE1-B)	Q9D051	38 937.3	6.41	4	161	68.30
4	Malate dehydrogenase, mitochondrial precursor (EC 1.1.1.37)	P08249	35 596.6	8.82	4	16	59.74
5	γ-Enolase (EC 4.2.1.11) (enolase 2)	P17183	47 165.7	4.99	4	14	58.05
6	Triosephosphate isomerase (TIM) (EC 5.3.1.1)	P17751	26 581.6	7.09	4	20	54.86
7	Creatine kinase, ubiquitous mitochondrial precursor (EC 2.7.3.2) (U-MtCK) (Mia-CK) (acidic-type mitochondrial creatine kinase)	P30275	47 004.0	8.39	4	18	52.85
8	Fumarate hydratase, mitochondrial precursor (EC 4.2.1.2) (fumarase) (EF-3)	P97807	54 371.1	9.11	3	10	44.68
9	Dihydrolipoyl dehydrogenase, mitochondrial precursor (EC 1.8.1.4) (dihydrolipoamide dehydrogenase)	O08749	54 212.6	7.97	3	10	43.63
10	Vacuolar ATP synthase subunit β, brain isoform (EC 3.6.3.14)	P62814	56 551.1	5.57	2	6	37.31
11	Pyruvate dehydrogenase E1 component subunit α, somatic form, mitochondrial precursor	P35486	43 231.9	8.49	3	11	34.73
12	L-Lactate dehydrogenase B chain (EC 1.1.1.27) (LDH-B)(LDH heart subunit)	P16125	36 441.3	5.70	3	11	34.39
13	Pyruvate carboxylase, mitochondrial precursor (EC 6.4.1.1)	Q05920	129 685.3	6.25	2	2	31.74
14	LL-Lactate dehydrogenase A chain EC 1.1.1.27) (LDH-A) (LDH muscle subunit)	P62259	29 174.1	4.63	3	13	30.28
15	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	P16858	35 679.0	8.45	2	8	26.18
16	2-Oxoglutarate dehydrogenase E1 component, mitochondrial precursor (EC 1.2.4.2) (α-ketoglutarate dehydrogenase)	Q60597	116 118.3	6.52	2	3	24.54
17	α-Enolase (EC 4.2.1.11)	P17182	47 009.9	6.36	2	7	22.75
18	Fructose-bisphosphate aldolase C (EC 4.1.2.13), brain-type aldolase	P05063	39 263.9	6.79	2	8	20.14
19	Glycogen phosphorylase, brain form (EC 2.4.1.1)	Q8C194	96 599.3	6.29	2	3	20.12
II Proteins involved in cytoskeleton formation and exocytosis (*n* = 13)							
1	Synaptotagmin-1	P46096	47 418.3	8.68	6	19	82.7
2	Syntaxin-binding protein 1 (Unc-18 homolog) (Unc-18A) (Unc-18-1)	O08599	667 569.1	6.50	6	15	79.91
3	AP-2 complex subunit β-1	Q9DBG3	104 583.2	5.22	5	10	69.37
4	AP-2 complex subunit α-1	P17426	107 664.7	6.63	5	6	68.88
5	Actin, cytoplasmic 2(γ-actin)	P63260	41 793.1	5.31	4	19	58.07
6	Spectrin α chain, brain	P16546	167 554.0	5.27	3	3	49.80
7	Dynamin-1 (EC 3.6.5.5)	P39053	97 803.3	7.61	3	4	41.88
8	Spectrin β chain, brain 1	Q62261	274 224.5	5.40	3	1	33.06
9	Rab GDP dissociation inhibitor α (Rab GDP)	P50396	50 521.9	4.96	2	8	32.38
10	Tubulin α-2 chain (α-tubulin 2)	P05213	50 151.9	4.94	2	7	26.93
11	Clathrin heavy chain	Q68FD5	191 557.7	5.48	2	2	26.66
12	AP-2 complex subunit mu-1	P84091	49 655.0	9.57	2	8	21.89
13	Glial fibrillary acidic protein, astrocyte (GFAP)	P03995	49 908.3	5.36	2	5	20.70
III Proteins involved in regulation of gene expression, cell division, and differentiation (*n* = 13)							
1	Dihydropyrimidinase-related protein 2 (DRP-2) (ULIP 2 protein)	O08553	62 277.9	5.95	11	35	182.87
2	Heterogeneous nuclear ribonucleoprotein A3	Q8BG05	39 652.2	9.09	8	22	137.85
3	Valosin-containing protein (VCP)	Q01853	89 177.1	5.14	8	16	124.17
4	Heterogeneous nuclear ribonucleoprotein Q	Q71MK9	69 633.0	8.68	6	13	79.54
5	Heterogeneous nuclear ribonucleoprotein K	P61979	50 976.5	5.39	5	19	75.62
6	Heterogeneous nuclear ribonucleoprotein A1	P49312	34 065.2	9.27	5	13	70.70
7	Transcriptional activator protein Pur-α (Purine-rich single-stranded DNA-binding protein α)	P42669	34 883.9	6.07	3	24	55.93

Table 3. Continued

No.	Protein name	Database accession number	MW, Da	p*I*	No. of peptides	Sequence coverage, %	Mill search score
8	Heterogeneous nuclear ribonucleoprotein L	Q8R081	60 123.6	6.65	3	7	49.06
9	RNA-binding protein FUS (Pigpen protein)	P56959	52 673.4	9.40	2	6	32.73
10	Cullin-associated NEDD8-dissociated protein 1	G6ZQ38	136 332.4	5.52	2	2	25.45
11	Interleukin enhancer-binding factor 2	Q9CXY6	43 062.4	5.19	2	9	24.29
12	Polyadenilate-binding protein 1	P29341	70 643.2	9.48	2	4	22.89
13	Heterogeneous nuclear ribonucleoprotein A/B	Q99020	30 831.4	7.69	2	10	19.39
IV Proteins involved in signal transduction and regulation of enzyme activity ($n = 8$)							
1	14-3-3 Protein ζ/δ (protein kinase C inhibitor protein 1) (KCIP-1) (SEZ-2)	P63101	27 771.3	4.73	10	38	152.28
2	14-3-3 Protein γ	P61982	28 171.5	4.80	4	20	55.73
3	14-3-3 Protein η	P68510	28 080.7	4.81	3	15	43.37
4	14-3-3 Protein θ	P68254	27 778.4	4.69	3	20	39.23
5	Guanine nucleotide-binding-protein β subunit 2-like 1	P68040	34 945.7	7.57	2	11	25.77
6	2′,3′-Cyclic-nucleotide 3′-phosphodiesterase (EC 3.1.4.37)	P16330	47 123.6	9.08	2	5	23.92
7	Serine/threonine-protein phosphatase 2A65 kDa regulatory subunit A, α isoform	Q76MZ3	65 191.8	5.00	2	5	23.48
8	14-3-3 Protein β/α	Q9CQV8	27 955.4	4.77	2	16	23.27
V Antioxidant enzymes and protective proteins ($n = 6$)							
1	Heat shock cognate 71 kDa protein (heat shock 70 kDa protein 8) heat shock-related 70 kDa protein 2	P63017	70 871.4	5.38	12	27	193.09
2	Stress-70 protein, mitochondrial precursor	P38647	73 528.7	5.91	3	6	34.92
3	Peroxiredoxin-5, mitochondrial precursor (EC 1.11.1.15) (Prx-V) (peroxisomal antioxidant enzyme) (thioredoxin reductase)	P99029	21 897.6	9.09	3	24	34.26
4	Superoxide dismutase [Mn], mitochondrial precursor (EC 1.15.1.1)	P09671	24 603.1	8.80	2	12	28.74
5	Peroxiredoxin-2 (EC 1.11.1.15) (thioredoxin peroxidase 1) (thioredoxin-dependent peroxide reductase 1)	Q61171	21 647.6	5.20	2	9	28.49
6	Glutathione *S*-transferase P1 (EC 2.5.1.18)	P19157	23 478.1	8.13	2	12	28.34
VI Proteins/enzymes involved in metabolism of nucleotides and amino acids and related processes ($n = 5$)							
1	Aspartate aminotransferase, mitochondrial precursor (EC 2.6.1.1) (transaminase A) (glutamate oxaloacetate transaminase 2)	P05202	47 411.6	9.13	5	19	65.74
2	Guanine deaminase (EC 3.5.4.3)	P38647	73 528.7	5.91	3	6	34.92
3	Pyridoxal kinase (EC 2.7.1.38)	Q8K183	35 015.4	5.88	2	7	32.80
4	Ribose-phosphate pyrophosphokinase I (EC 2.7.6.1)	Q9D7G0	34 717.3	6.57	2	10	28.87
5	4-Aminobutyrate aminotransferase, mitochondrial precursor (EC 1.15.1.1)	P61922	56 452.2	8.35	2	5	28.02

A low coincidence of proteins from the group of mouse and rat proteins/enzymes involved in cell signaling (and possibly some others) may account for some differences in responsiveness of rats and mice to isatin effects. For example, Bhattacharya *et al.* [9, 63] reported about anxiogenic activity of low doses of isatin in the open-field and elevated plus -maze tests in albino mice, whereas Abel [64] failed to observe such effect of isatin in the open-field test in Sprague Dawley rats.

Interaction of some of the isatin-binding proteins with isatin has been validated in the optical biosensor study that employed a Biacore 3000 optical biosensor and 5-aminocaproyl-isatin as the affinity ligand. Previously, another isatin analogue, 5-aminoisatin, was used as the affinity ligand for analysis of isating-binding activity in subcellular fractions of rat brain, liver, kidney, and heart [65] and for the study of interaction of purified glyceraldehyde-3-phosphate dehydrogenase with isatin [17]. Therefore, it was interesting to compare interaction of the selected individual proteins not only with 5-aminocaproyl-isatin but also with 5-amino-isatin. The results shown in Table 5 clearly indicate that all the studied proteins exhibited specific interaction not only with 5-aminocaproylisatin used for the fishing of rat and mouse brain isatin-binding proteins but also with 5-aminoisatin. Thus, it appears that both affinity ligands may be used for effective fishing of isatin-binding proteins in different tissues and cells. However, the nature of the spacer between the amino-group used for covalent

Table 4. Proteomic identification of mouse brain proteins nonspecifically bound to control Sepharose

No.	Protein name	Database accession number	MW, Da	p*I*	No. of pep-tides	Sequence coverage, %	Mill search score
1	Keratin, type II cytoskeletal 1	P04104	65 606.2	8.39	3	3	48.59
2	Keratin, type II cytoskeletal 15	Q922U2	61 767.0	7.59	3	5	45.02
3	Keratin, type II cytoskeletal 73	Q6NXH9	58 911.8	8.35	2	4	35.89
4	Keratin, type II cytoskeletal 79	Q8VED5	57 552.6	7.55	2	4	33.23
5	Keratin, type II cytoskeletal 6B	Q9Z331	60 322.6	8.50	2	4	33.04
6	Keratin, type II cytoskeletal 75	Q8BGZ7	59 740.8	8.46	2	4	33.04
7	Keratin, type II cytoskeletal 6A	P50446	59 335.4	8.04	2	4	33.04
8	Keratin, type II cytoskeletal 1B	Q6IFZ6	61 359.0	7.73	2	3	30.98
9	Keratin, type I cytoskeletal 42	Q6IFX2	50 133.3	5.09	2	4	30.02
10	Keratin, type I cytoskeletal 10	P02535	57 770.1	5.04	2	3	29.31
11	Keratin, type II cytoskeletal 8	P11679	54 565.6	5.70	2	4	28.18
12	Keratin, type I cytoskeletal 15	Q61417	49 137.8	4.79	2	3	26.57
13	Keratin, type I cytoskeletal 17	Q9QWL7	48 162.1	5.00	2	4	26.57
14	Keratin, type I cytoskeletal 19	P19001	44 542.0	5.28	2	4	26.57
15	Tubulin α-1A chain	P68369	50 135.9	4.94	2	5	23.12
16	Tubulin α-1B chain	P05213	50 151.9	4.94	2	5	23.12
17	Tubulin α-1C chain	P68373	49 909.6	4.96	2	5	23.12
18	Keratin, type II cytoskeletal 71	Q9R0H5	57 382.8	6.61	1	2	19.00
19	Keratin, type II cytoskeletal 7	Q9DCV7	50 709.0	5.67	1	2	16.89

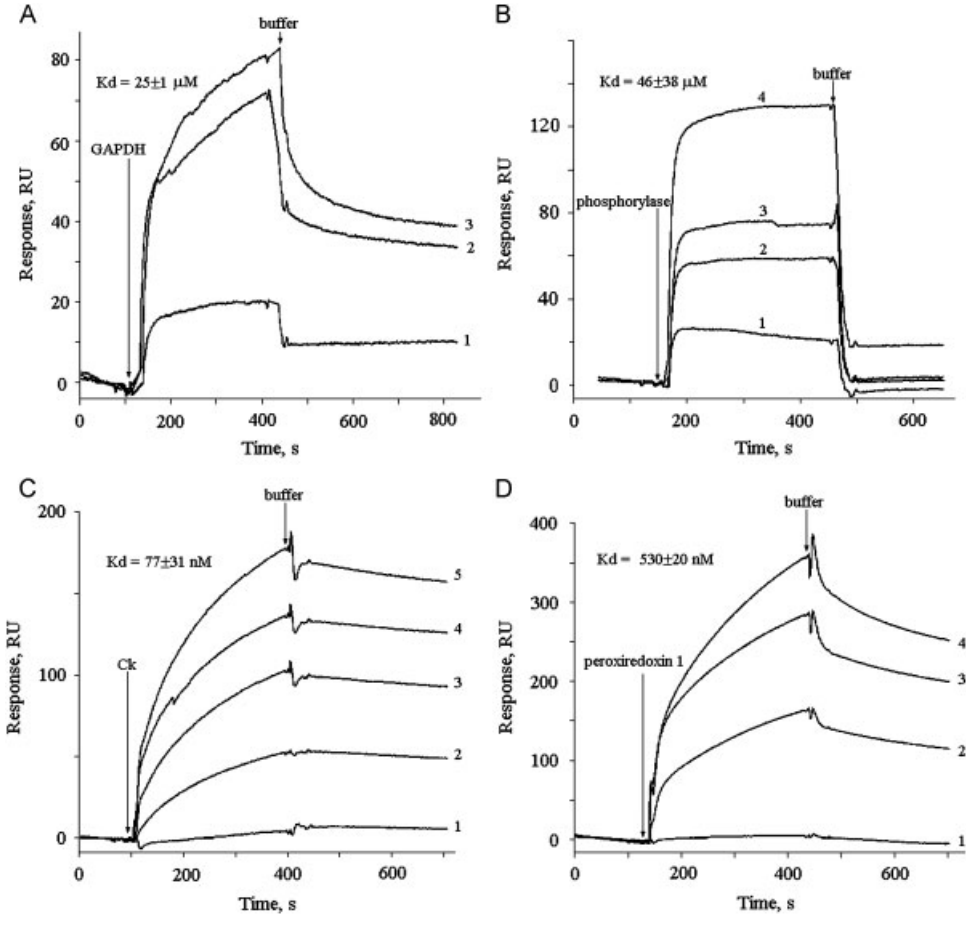

Figure 1. Interaction of individual enzymes with immobilized 5-aminocaproyl-isatin. Concentrations of added proteins are shown as micromolar. (A) (GAPDH): 1–10 μM; 2–20 μM; 3–50 μM; (B) (phosphorylase): 1–10 μM; 2–20 μM; 3–30 μM; 4–50 μM; (C) (creatine kinase): 1–0.1 μM; 2–1 μM; 3–2 μM; 4–3 μM; 5–4 μM; (D) (peroxiredoxin 1): 1–0.1 μM; 2–0.5 μM; 3–1.5 μM; 4–2.5 μM.

Table 5. Kinetic parameters of proteins binding to immobilized 5-aminocaproyl-isatin or 5-aminoisatin

Immobilized ligand	Protein	k_{on}, M^{-1}/s	k_{off}, s^{-1}	K_d, M
5-Aminocaproyl-isatin	Glyceraldehyde-3-phosphate dehydrogenase	$(2.16\pm0.09)\times10^{3}$	$(5.4\pm0.1)\times10^{-2}$	$(2.5\pm0.1)\times10^{-5}$
	Creatine phosphokinase	$(1.30\pm0.02)\times10^{3}$	$(1.0\pm0.4)\times10^{-4}$	$(7.7\pm3.1)\times10^{-8}$
	Peroxiredoxin	$(4.50\pm0.08)\times10^{3}$	$(2.4\pm0.1)\times10^{-3}$	$(5.3\pm0.2)\times10^{-7}$
	Glycogen phosphorylase[a)]	–	–	$(4.6\pm3.8)\times10^{-5}$
5-Aminoisatin	Glyceraldehyde-3-phosphate dehydrogenase	$(3.9\pm0.2)\times10^{3}$	$(1.10\pm0.04)\times10^{-2}$	$(2.8\pm0.2)\times10^{-6}$
	Creatine phosphokinase	$(1.33\pm0.02)\times10^{3}$	$(3.8\pm0.5)\times10^{-4}$	$(2.9\pm0.4)\times10^{-7}$
	Peroxiredoxin	$(2.84\pm0.05)\times10^{4}$	$(2.5\pm0.1)\times10^{-3}$	$(8.8\pm0.4)\times10^{-8}$
	Glycogen phosphorylase[a)]	–	–	$(3.0\pm0.6)\times10^{-5}$

k_{on}, association rate constant; k_{off}, dissociation rate constant.

a) Due to low affinity binding the K_d values for glycogen phosphorylase have been calculated using the steady-state affinity option of the Biacore software.

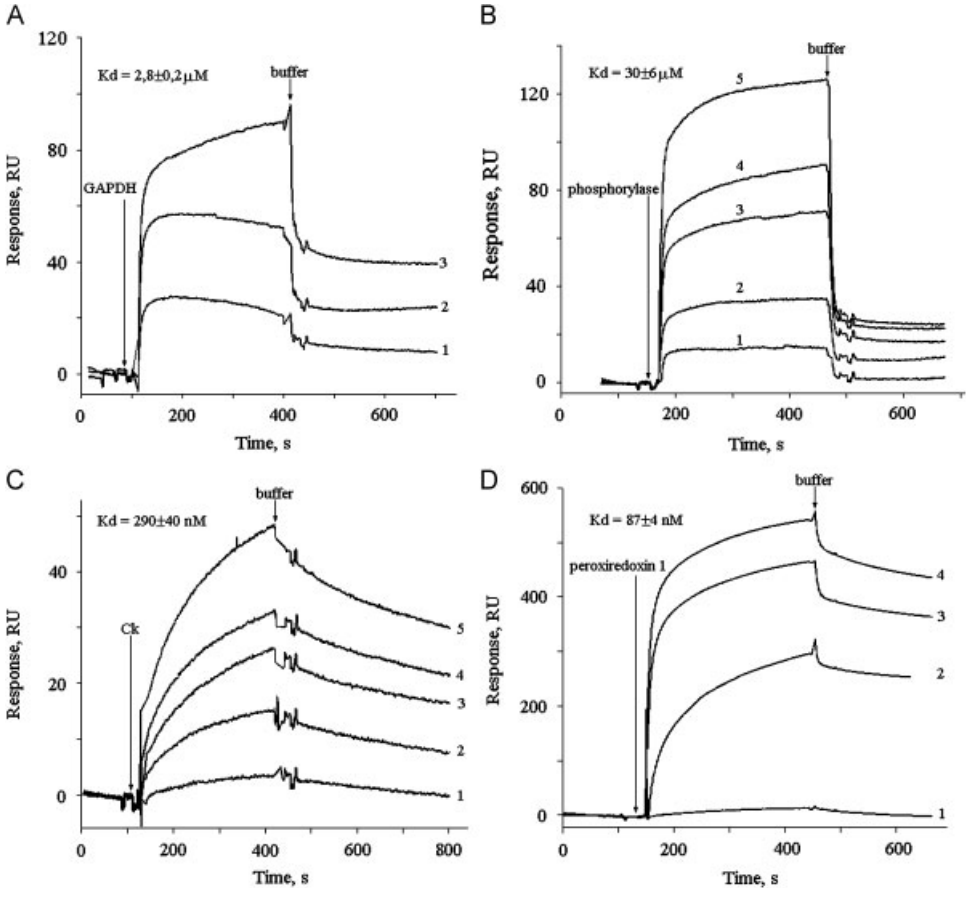

Figure 2. Interaction of individual enzymes with immobilized 5-aminoisatin. Concentrations of added proteins are shown as micromolar. (A) (GAPDH): 1–5 μM; 2–10 μM; 3–20 μM; (B) (phosphorylase): 1–5 μM; 2–10 μM; 3–20 μM; 4–30 μM; 5–50 μM; (C) (creatine kinase): 1–0.1 μM; 2–1 μM; 3–2 μM; 4–3 μM; 5–4 μM; (D) (peroxiredoxin 1): 1–0.1 μM; 2–1 μM; 3–1.5 μM; 4–2.5 μM.

immobilization of isatin analogues to the carboxymethylated dextran chip and the isatin moiety may influence the interaction of a particular target with isatin. Four proteins tested in this study exhibited different preferences towards these two analogues. Glycogen phosphorylase demonstrated basically the same affinity to 5-aminocaproyl-isatin and 5-aminoisatin (K_d values of 46 ± 38 *versus* 30 ± 6 μM, respectively). The affinity of GAPDH and peroxiredoxin was higher with 5-amino-isatin as the affinity ligand (K_d values of 2.8 ± 0.2 μM and 87 ± 4 nM than that with 5-aminocaproyl-isatin (K_d values of 25 ± 1 μM and 530 ± 20 nM), whereas creatine phosphokinase exhibited a higher affinity towards immobilized 5-aminocaproyl-isatin (77 ± 31 nM) than 5-aminoisatin (290 ± 40 nM).

Table 6. Percent of isatin-binding proteins common for mouse and rat brain

Group of proteins	Mouse brain	Rat brain
I	7/19 (37%)	7/15 (47%)
II	8/13 (62%)	8/19 (42%)
III	3/14 (21%)	3/17 (18%)
IV	1/8 (13%)	1/7 (14%)
V	1/6 (17%)	1/6(17%)

Such behaviour of individual proteins towards various analogues should be taken into consideration because the most sensitive previously known targets of isatin (natriuretic peptide receptor guanylate cyclase, soluble nitric oxide stimulated guanylate cyclase, and monoamine oxidase B) have not been identified as the isatin-binding proteins either in rat or in mouse brains. On the one hand, the resolution of membrane-bound proteins (including plasma membrane

Table 7. Isatin-binding proteins common for rat and mouse brain and their changes in pathology

Protein name	Altered function in pathology	Reference
I Proteins/enzymes involved in energy generation and carbohydrate metabolism		
Mitochondrial aconitase	Forms associates of high molecular weight and exhibits loss of catalytic activity in Huntington's Disease brain	[27]
Creatine kinase B-type	Exhibits oxidative modification and decreased activity in Alzheimer's disease brain	[28]
Triosephosphate isomerase (TIM)	Undergoes regiospecific oxidative modification in Alzheimer's disease brain	[29] [30]
Dihydrolipoamide dehydrogenase	Decreased activity was found in several neurodegenerative to diseases, lack of this enzyme in mice increased vulnerability to MPTP and other toxins	[31]
Glyceraldehyde-3-phosphate dehydrogenase	Undergoes regiospecific oxidative modification in Alzheimer's disease brain and after *in vivo* injection of amyloid β-peptide (1–42) into rat brain; target for neuroprotective drugs; initiates apoptotic cascades	[29, 32–35]
α-Enolase	Undergoes regiospecific oxidative modification in Alzheimer's disease brain	[29, 30]
Glycogen phosphorylase	Undergoes regiospecific oxidative modification in Alzheimer's disease brain	[29]
II Proteins involved in cytoskeleton formation and exocytosis		
Syntaxin binding protein (Unc-18 protein homologue)	Undergoes oxidative modification	[36]
Spectrin α chain, brain	Its cleavage accompanies apoptosis	[37, 38]
Dynamin-1	Dynamin and dynamin-related proteins (*e.g.* OPA-1) are involved in pathology and protection against neurodegenerative diseases	[39–41]
Spectrin β 1 chain, brain	Its impairments affect proteins involved in glutamate signaling; induce irreversible fragmentation of the Golgi apparatus typical for disorders like Alzheimer's disease	[42, 43]
Tubulin α-2 chain	Undergoes modification after depletion of reduced GSH in the brain	[44]
Clathrin heavy chain	Binds to huntingtin, which is involved in pathogenesis of Huntington's Disease	[45]
Glial fibrillary acidic proteins (Gfap)	Involved in inflammatory changes in the brain of animals with experimental models of Alzheimer's disease; brain injury-associated biomarker, involved in astrogilosis	[46–48]
Valosin-containing protein	A member of ATPases superfamily; it has an antiapoptotic function and is co-localized with protein aggregates in neurons of patients with neurogenerative disorders	[49–51]
III Proteins involved in regulation of gene expression, protein folding, and cell cycle and differentiation		
Dihydropyrimidinase-related protein 2 (DLR-2) (known as TOAD-64)	Upregulated after focal cerebral ischemia; increased oxidation in Alzheimer's disease brain	[52, 53]
Heterogeneous nuclear ribonucleoprotein A2	One of the inclusion-associated proteins revealed in post-mortem brain tissue of patients with fragile X-associated tremor ataxia syndrome. HnRNP A2 could serve as a mediator of the expanded-repeat FMR1 mRNA in FXTAS	[54]
Heterogeneous nuclear ribonucleoprotein A1	One of apoptosis-associated proteins, cleaved by caspase-3; undergoes stress-induced cytoplasmic accumulation	[55, 56]
IV Proteins involved in cell signalling and signal transduction		
2′3,′-Cyclic nucleotide phosphodiesterase	The enzyme inhibition is accompanied by a neuroprotective effect in several models of neurotoxicity	[52, 57]

Table 7. Continued

Protein name	Altered function in pathology	Reference
V Antioxidant and protective enzymes		
Peroxiredoxin 2	Undergoes regioselective oxidative modification in early Alzheimer's disease brain and is involved in the neuroprotective effects of pituitary adenylate cyclase activating polypeptide	[30, 58]

receptor guanylate cyclase and monoamine oxidase B of the outer mitochondrial membranes) represents a serious methodological problem in modern proteomics (*e.g.* [66–68]). On the other hand, it is possible that the affinity of at least some of the ''missed'' proteins towards the isatin analogues employed in our studies is not enough for the affinity fishing of these isatin-binding proteins. Taking into consideration the K_d values of 30–50 μM obtained in the case of glycogen phosphorylase (Fig. 1 and 2) as the lowest affinity limit for protein fishing from tissue preparations and the K_i values of 20 μM [69] for inhibition of mitochondrial monoamine oxidase one may assume that monoamine oxidase ligand binding still needs a better isatin analogue. This suggestion seems to be reasonable if we take into consideration that modification of the isatin moiety at the 5th position dramatically decreased the potency of monoamine oxidase B inhibition [65, 69, 70].

The results of the present study provide convincing evidence for the existence of a large group of isatin-binding proteins in both rat and mouse brain. Specific interaction of four individual proteins with the isatin analogues immobilized on the carboxymethylated dextran chip of the Biacore biosensor suggests that the other proteins identified in this study may also exhibit interaction with isatin. Such a possibility seems even more reasonable, if we take into consideration the fact that the range of brain concentrations of the proteins identified in this study is comparable with the levels of four individual proteins tested in the optical biosensor experiments [71]. In all cases their content in brain cells (ranged from nanomole to picomole *per* 1 mg of protein) [71] is significantly lower than brain isatin concentrations (reaching micromolar range in hippocampus, cerebellum, and striatum) [3]. This also implies the possibility of interaction between isatin and isatin-binding proteins identified in this study.

Isatin attracts much interest due to its role as an endogenous regulator of various neurochemical and physiological processes [1, 2] and also due to the potential pharmacological actions of isatin itself and its many analogues [2–5]. The isatin moiety is often present as a fragment in diverse compounds with a range of pharmacological actions, including inhibition of apoptosis [72, 73] and anticonvulsant action [74]. Some of these isatin compounds have been suggested to be potentially useful in treating CNS diseases such as epilepsy, Parkinsonism, and senile dementia [4].

Although the biological importance of the isatin interaction with the identified proteins requires further investigation, it should be noted that glyceraldehyde-3-phosphate dehydrogenase, which is considered as a putative target for neuroprotective drugs [17, 75, 76], exhibits various biological activities including interaction with cytoskeletal proteins [77]. Other glycolytic enzymes, isatin-binding proteins, identified in this study (triose phosphate isomerase, enolase) appear to exhibit various interactions with other cytosolic (cytoskeletal) proteins as well [77–79]. Thus, it is possible that the known antiproliferative and proapoptotic effects of isatin [80, 81] may also be associated with rearrangement of cytoskeleton and/or cytoskeleton-associated proteins.

Thus, we suggest that altered isatin concentrations seen in stress [7] and Parkinson's disease [82] may influence the interaction of isatin-binding proteins with cytoskeletal structures, important for the manifestation or attenuation of neurodegenerative and psychiatric disorders. Recent evidence suggests that isatin improves symptoms of Parkinsonism studied in different experimental models [83].

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The authors have declared no conflict of interest.

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