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Adenosine 5′-triphosphate binding to bovine serum albumin

Seiji Takeda [a], Seiji Miyauchi [a], Hitoshi Nakayama [b], Naoki Kamo [a,*]

[a] *Laboratory of Biophysical Chemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, 060, Japan*

[b] *Laboratory of Radiopharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Kumamoto University, Kumamoto 862, Japan*

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Abstract

Binding of ATP to bovine serum albumin was shown by ultrafiltration and NMR. The binding was pH dependent. Scatchard analysis revealed that at pH 5.4, 6.4 and 7.4, dissociation constant K_d was 13, 40 and 120 μM, respectively, and no binding was observed at pH 8.4. The binding stoichiometry was 1:1 for all pH. Dimer of BSA did not bind ATP. From chemical shifts of ^{31}P-NMR, K_d was estimated to be 15 μM at pH 5.4, which is very close to that determined by ultrafiltration. While adenosine did not interfere with the binding, GTP, dCTP, ADP, UTP, AMP, phosphate and pyrophosphate were competitive inhibitors and their inhibition constants K_i were 25, 32, 36, 50, 130, 1000 and 186 μM, respectively. Fatty acids such as lauric acid and palmitic acid did not interfere with the binding. Warfarin was a non-competitive inhibitor. Cl^- competitively inhibited the binding, and the inhibition constant was 20 mM. The dissociation constants of the Cl^- binding were reported to be 0.42 mM for the first binding site, 10-5 mM for the second and 303-143 mM for the third [G. Scatchard, W.T. Yap, J. Am. Chem. Soc., 86 (1964) 3434; G. Scatchard et al., J. Am. Chem. Soc. 79 (1957) 12]. This suggests that the ATP binding site may be the second Cl^- binding site. © 1997 Elsevier Science B.V.

Keywords: ATP; BSA; Competitive inhibition by nucleotide; Competitive inhibition by phosphate and pyrophosphate; Cl^--binding; Ultrafiltration

1. Introduction

Serum contains proteins that account for 6 to 8% of the content and about half of these are albumin. Serum albumin is thus the most abundant protein in blood plasma. Albumin works as a depot protein and a transport protein for numerous endogenous and exogenous compounds. Endogenous substrates that bind to albumin include fatty acids, eicosanoids, bile acids, steroids, bilirubin, hematin, L-tryptophan, L-thyroxine, 25-OH-vitamin D_3, folate, ascorbate, various metal ions and chloride. Among the exogenous substrates are salicylate, warfarin, digitoxin, indomethacin, bromphenol blue, iophenoxate, sulfobromophthalein, diazepan, ibuprofen, chlorpromazine, imipramine, quinidine, procain and lidocain. Thus, there are many compounds that bind to serum albumin irrespective of their bearing charges [1,2]

Although BSA (bovine serum albumin) is often added to experiments in ATP (adenosine 5′-triphosphate)-dependent systems such as ion-transporting ATPase, proteases and others, the role of BSA is not essential but is instead to increase the solubility or stability of chemicals [3–8]. For Na^+–K^+–ATPase [9] or Hsp90 [10] study, BSA was used as a control

* Corresponding author.

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protein because it is believed not to interact with ATP; careful examination of these earlier data, however, indicated that ATP did bind to BSA. Bauer et al. [11] reported that a spin-labelled ATP actually bound to BSA, stating, however, that other investigators failed to detect the interaction by differential scanning calorimetry. Therefore, the question of whether ATP binds to BSA remains to be answered. In this paper, we present data showing firm ATP binding to BSA with a stoichiometry of 1:1. The dissociation constant was 13 μM at acidic pH (5.4), but was increased to 120 μM at neutral pH (7.4) and was not measurable in alkaline solutions. The binding was competitive with Cl^-. These results were obtained by the ultrafiltration method. The interaction between ATP and BSA was also examined by NMR (nuclear magnetic resonance).

2. Materials and methods

2.1. Chemicals

Chemicals were of the highest commercial quality. ATP, GTP (guanosine 5′-triphosphate), UTP (uridine 5′-triphosphate), dCTP (deoxycytidine 5′-triphosphate), flufenamic acid, digitoxin, lauric acid, palmitic acid, MES (2-morphinoethanesulfonic acid) and Tris (2-amino-2-hydroxymethyl-1, 3-propanediol) were purchased from Wako Chemical (Osaka, Japan), and warfarin from Sigma (St. Louis, MO). [γ-^{32}P]-ATP (185 MBq/ml) was obtained from New England Nuclear (Boston, MA).

2.2. Purification of BSA

BSA (Fr. V) was obtained from Sigma and purified (defatted) using activated charcoal according to Chen [12] or Sogami and Foster [13]. The purified (defatted) BAS was stored at 4°C and used within 3 weeks. To remove the dimer, purified (defatted) BAS was further applied to gel-filtration with Sephacryl S-200 (Pharmacia, Uppsala, Sweden) column (120 × 0.9 cm) pre-equilibrated with 0.1 M phosphate buffer (pH 7.4) and eluted with the same buffer. The weight ratio of monomer to dimer was approximately 10:1, hence the mole ratio of dimer was ca. 5%.

2.3. Estimation of mercaptalbumin (BMA) content

BSA has only one free cysteine residue (Cys-34). Some fraction of this cysteine is usually occupied by another cysteine or glutathione. The BSA molecule containing the free cysteine residue is called mercaptalbumin. To estimate the mercaptalbumin content, defatted BSA was dissolved in 25 mM Tris–MES buffer to give 200 μM, and titrated with PCMB (parachloromercury benzoate) by the described method [14]. The titration yielded 24% of mercaptalbumin.

2.4. Ultrafiltration

A BSA solution (200–500 μM) containing an appropriate concentration of ATP and [γ-^{32}P]-ATP (usually 100–190 kBq) was incubated at 25°C for 15 min, then 1 ml was applied to the upper chamber of an ultrafiltration cassette (centrifree MPS-3, Grace Japan, Tokyo), and centrifuged at 3000 rpm (18-3 Hitachi rotor, Hitachi) for 3 min at the same temperature. BSA concentration was determined by the absorbance at 279 nm using ${}^{1\%}_{1\ \mathrm{cm}}E = 6.67$ at pH 7 [15]. ATP concentration was determined with the absorbance at 259 nm using $\epsilon^{\mathrm{mM}}_{259} = 15.4\ \mathrm{cm}^{-1}$. An aliquot of 100 μl was taken from the filtrate to measure the radioactivity with a liquid scintillation counter (Packard 1600 TR) which gave the free concentration of ATP. Prior to the centrifugation, the same volume had been taken from the ATP–BSA solution to measure the radioactivity that gave the total concentration of both free and bound ATP. From these two radioactivities, ATP concentrations of both free, C_f and bound, C_b were calculated.

2.5. Analysis of data

When ATP was used as a sole ligand, binding data were treated with the Scatchard plot [16]:

$$\frac{\bar{v}}{C_f} = \frac{n}{K_d} - \frac{\bar{v}}{K_d} \qquad (1)$$

where $\bar{v}$ = average number of bound ATP molecules per BSA molecule, C_f = free ATP concentration, n = maximum number of binding sites per BSA molecule, and K_d = dissociation constant, respectively.

When ATP and another compound were used as ligands to examine whether the binding was competitive to one site, data were analyzed according to Kragh-Hansen [2]. In brief, if an inhibitor (denoted by I) inhibits *competitively* the ATP binding to BSA, the following equations hold:

$$\bar{\nu}_{ATP} = \frac{n \cdot K_{ATP} \cdot F_{ATP}}{1 + K_{ATP} \cdot F_{ATP} + K_I \cdot F_I} \quad (2)$$

$$\bar{\nu}_I = \frac{n \cdot K_I \cdot F_I}{1 + K_{ATP} \cdot F_{ATP} + K_I \cdot F_I} \quad (3)$$

where $\bar{\nu}_r$ = number of molecule r bound per BSA molecule (r = ATP or I), K_{ATP} = binding constant of ATP to BSA (which equals a reciprocal of K_d that can be estimated from Eq. (1)), F_r = the concentration of free r and K_I = inhibition constant of I, respectively. Using mass conservation for I and the above two equations, F_I can be calculated. In addition, we can obtain an equation for $\bar{\nu}_I$ that contains only measurable quantities and K_{ATP} ($= 1/K_d$) as follows:

$$\bar{\nu}_I = n - \bar{\nu}_{ATP} \frac{1 + K_{ATP} \cdot F_{ATP}}{K_{ATP} \cdot F_{ATP}} \quad (4)$$

Note that $\bar{\nu}_{ATP}$ and F_{ATP} were obtainable by the ultrafiltration method using [γ-^{32}P]-ATP.

The ratio of Eqs. (2) and (3) gives the following equation:

$$\frac{\bar{\nu}_{ATP}}{\bar{\nu}_I} = \frac{K_{ATP}}{K_I} \frac{F_{ATP}}{F_I} \quad (5)$$

Eq. (5) describes that if ATP and I are in competition with the same binding site, the plot of $\bar{\nu}_{ATP}/\bar{\nu}_I$ against F_{ATP}/F_I should be linear and K_I is calculable from the proportional constant. If the plot does not give the linear relationship, the inhibition is not competitive (non-competitive).

2.6. Chemical shift of ^{31}P-NMR

All ^{31}P-NMR measurements were conducted using a Bruker MSL300 spectrometer operated at 121 MHz. BSA was dissolved in D_2O–H_2O mixture (mole ratio of 1:1) buffered at pH 5.4 with 25 mM MES–Tris to give 650 μM where D_2O was used as an internal lock. Inorganic phosphate solution at pH 10.0 was used as an external standard. There are two environments for ATP in a BSA solution: a bound and a free environment. When ATP exchanges fast enough between these two environments, resonance peaks that characterize each of the environments coalesce to a single resonance. The observed chemical shift is a weighted contribution from these two environments, from which we can estimate the dissociation constant [17].

2.7. Spin–spin relaxation time (T_2) of ^{1}H-NMR

^{1}H-NMR measurements were conducted with a Bruker MSL400 operated at 400 MHz using CPMG (Carr–Purcell–Meiboom–Gill) pulse sequence [17]. A 10.0 μs of $\pi/2$ pulse was used and the time between successive pulses was 1500 ms (T_1 = 300 ms). Data were obtained from 32-time average with the window of 0.5 Hz.

Measurements were made when ATP was added to give a final concentration of 4.9 mM and 10.1 mM with BSA being 634 μM. BSA was dissolved in D_2O buffered with 10 mM MES–NaOD at pH 5.4, and the sample temperature was maintained at 20°C. The carrier frequency was placed at the HDO resonance in all experiments. Data obtained were analyzed by the following equation [18,19]:

$$\frac{1}{T_2} = \frac{1}{T_{2f}} + P_b \left(\frac{1}{T_{2b}} - \frac{1}{T_{2f}} \right) \quad (6)$$

where T_{2f} and T_{2b} stand for T_2 values of free and bound ATP, respectively. In this equation, P_b is the fraction of bound to total ATP and calculated using K_d values obtained from the ultrafiltration.

3. Results

3.1. ATP binding to BSA

First, we measured the binding in solutions without buffers to exclude the possibility that salts used for the buffer inhibited the ATP-binding. After adequate dialysis of purified (defatted) BSA against pure water, pH of BSA solutions (final concentration

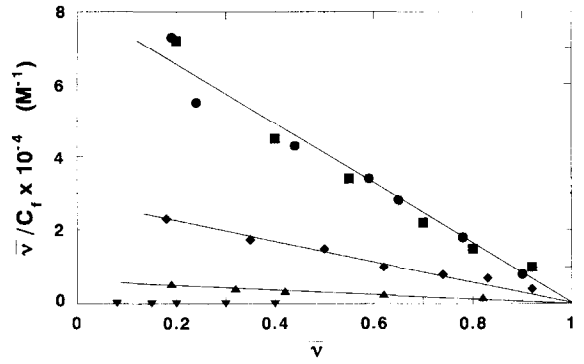


Fig. 1. Scatchard plot of ATP binding to BSA at varying pH. ●, pH 5.4 without buffer; ■, pH 5.4 buffered with 25 mM MES–Tris; ◆, pH 6.4 without buffer; ▲, pH 7.4 without buffer. Defatted and monomer BSA of 350 μM was used. ▼, dimer BSA at pH 5.4 without buffer. Temperature was 25°C.

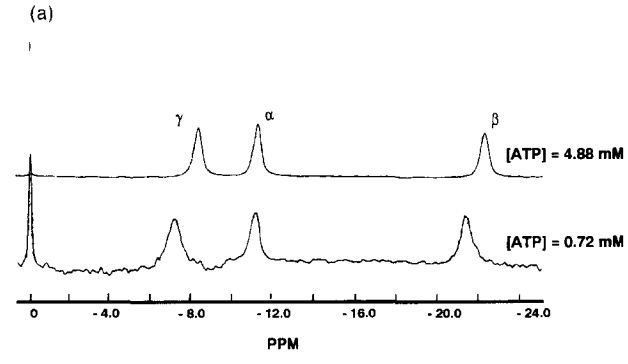
of 350 μM) was adjusted to 5.4, 6.4, 7.4 and 8.4 with minimal amounts of 1 M NaOH, followed by measurements of the ATP binding. Scatchard plots are shown in Fig. 1, and reveal that (1) ATP binds to BSA at one site/binding site per BSA, and that (2) the binding is pH-dependent. We tried various buffers and found that Cl^- interfered with the binding (see later) and that MES–Tris buffer had essentially no effect (see Fig. 1). The dissociation constant, K_d was calculated as 13 μM (at pH 5.4), 40 μM (at pH 6.4), 120 μM (at pH 7.4) and at pH 8.4 no binding was observed. The following experiments were hence conducted in the presence of 25 mM MES–Tris or MES–NaOH buffer.

No CD (circular dichroism) signals (205 nm–250 nm) were detected by the ATP binding to BSA, implying that no large conformational change occurred.

Fig. 1 also shows that ATP does not bind to the BSA dimer even at pH 5.4. Since, as mentioned in Section 2, the mole ratio of dimer was 5%, the data described below were obtained using BSA without removal of the dimer.

3.2. Chemical shift of γ-^{31}P-ATP NMR spectrum

^{31}P-NMR spectra were measured by addition of a concentrated ATP solution (final concentration of 350 μM–17 mM) to a BSA solution (650 μM in 25 mM MES–Tris buffer at pH 5.4). Fig. 2a shows spectra obtained at ATP concentrations of 720 μM and 4.88 mM. In Fig. 2, chemical shifts of γ-^{31}P NMR signals are plotted against ATP concentration since the γ-^{31}P chemical shift was the largest. Assuming that chemical shifts are simply weighted contributions from two populations, we estimated the values of K_d and the chemical shift of the bound ATP with the aid of non-linear regression. We obtained 15 ± 5 μM of K_d and 1.65 ppm (200 Hz) of the chemical shift of the bound ATP with the reference of free ATP. The solid line in this figure represents the calculated values using these values, showing good agreement with those observed. The



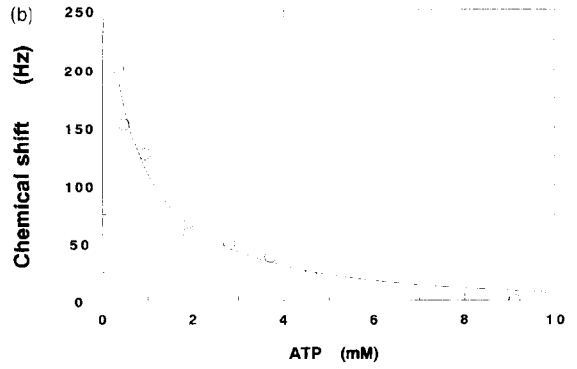


Fig. 2. (a) Typical ^{31}P-NMR spectra of BSA–ATP solution. ATP concentrations were 720 μM (lower trace) and 4.88 mM (upper). BSA was 650 μM dissolved in 25 mM MES–Tris at pH 5.4. For experimental conditions, see text. (b) Plot of chemical shifts of γ-^{31}P-ATP against ATP added. Varying concentrations of ATP (final concentration of 350 μM–17 mM) was added to BSA solution (650 μM in 25 mM MES–Tris buffer at pH 5.4), followed by NMR measurements. The chemical shift at 17 mM ATP was not plotted because it was almost equal to 9.1 mM ATP. The shifts are thought to be weighed contributions between free and bound populations, since resonance peaks that characterize each population coalesce to a single resonance as shown in (a). The solid curve was drawn assuming $K_d = 15$ μM.

K_d value of 15 μM is approximately equal to 13 μM (at pH 5.4) as determined from the ultrafiltration experiment.

3.3. Inhibition of the ATP binding by compounds whose chemical structure is analogous or common to ATP

First, adenosine was examined in the presence of 1 mM adenosine. ATP binding to BSA (250 μM) was measured and no effect on the Scatchard plot was observed (data not shown). Next, interference of ADP and AMP on the ATP binding was analyzed according to the Kragh-Hansen equation [2] and results are shown in Fig. 3, where $\bar{v}_{ATP}/\bar{v}_I$ was plotted against F_{ATP}/F_I (I = AMP, ADP and ATP). Concentrations of both ADP and AMP were 200 μM. BSA concentration was 220 μM and the solution was buffered with 25 mM MES–Tris at pH 6.0.

The data shown by open circles were those obtained when ATP itself was treated as an inhibitor; prior to experiments BSA was equilibrated with 200 μM ATP, followed by addition of appropriate concentrations of ATP together with radioactive ATP to measure the ATP binding. Theoretically, the slope of such experiments should be unity, and this is in fact the case, implying that our measurement is precise enough. Since the linear relations were obtained,

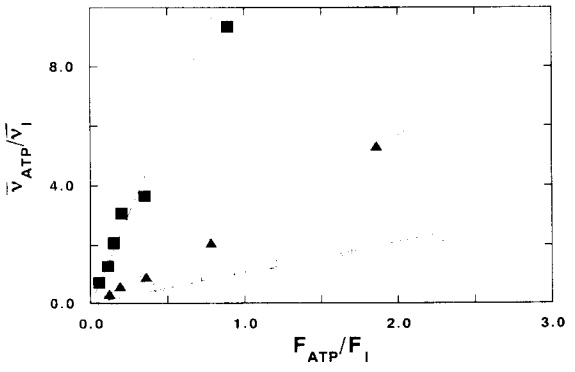


Fig. 3. ADP and AMP are competitive inhibitors for ATP-binding to BSA. According to Kragh-Hansen [2], $\bar{v}_{ATP}/\bar{v}_I$ was plotted against F_{ATP}/F_I. Notations of $\bar{v}_{ATP}$, $\bar{v}_I$, F_{ATP} and F_I are referred to in the text. The BSA solution was 220 μM buffered with 25 mM MES–Tris at pH 6.0. Temperature was 25°C. Data shown by ○ are the control experiment where ATP itself was treated as an inhibitor. ▲, in the presence of 200 μM ADP; ■, AMP of the same concentration.

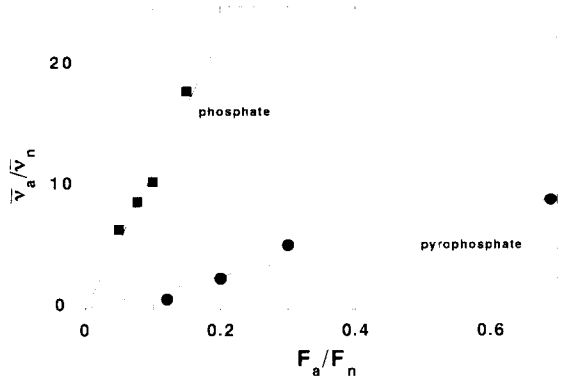


Fig. 4. Phosphate and pyrophosphate are also competitive inhibitors for ATP-binding to BSA. The BSA solution was 220 μM buffered with 25 mM MES–Tris at pH 5.4. Temperature was 25°C. ■, in the presence of 1 mM phosphate; ●, in the presence of 250 μM pyrophosphate.

ADP and AMP are competitive inhibitors with $1/K_I$ being 36 μM of ADP and 130 μM of AMP. Therefore, the binding to BSA increased in the following order: AMP < ADP < ATP.

The similar analysis was conducted for pyrophosphate and phosphoric acid, where concentrations were 250 μM and 1 mM, respectively, and BSA concentration was 220 μM buffered with 25 mM MES–Tris at pH 5.4. The results in Fig. 4 show that both are competitive inhibitors. Fig. 5 illustrates that UTP, dCTP and GTP are also competitive inhibitors. In this experiment, the concentrations of inhibitors were 100, 250, 450 or 1000 μM, and varying concentra-

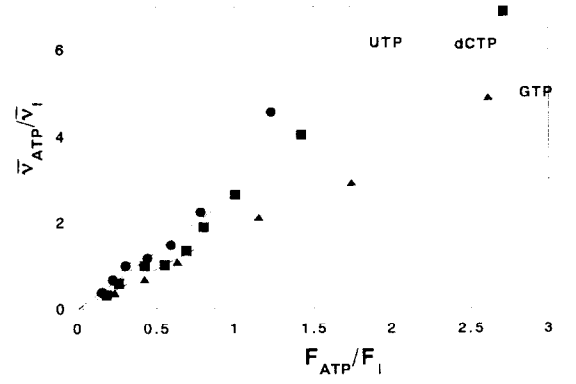


Fig. 5. UTP, dCTP and GTP are also competitive inhibitors. The BSA solution was 220 μM buffered with 25 mM MES–Tris at pH 5.4. Temperature was 25°C. ●, UTP; ■, dCTP; ▲, GTP. See concentrations of inhibitors and ATP in text.

Table 1
Inhibition constants of various compounds that have chemical structure analogous or common to ATP

Compound	GTP	dCTP	UTP	ADP	AMP	Pi	PPi
$1/K_I$ (μM)	25	32	50	36	130	1000	186

Pi and PPi stand for phosphate and pyrophosphate, respectively.

tions of ATP were added so as to give the values on the abscissa. Various combinations of concentrations between inhibitors and ATP gave a single straight line. The inhibition constants of these compounds are listed in Table 1 together with those for ADP and AMP.

3.4. Spin–spin relaxation time (T_2) of proton

When ATP interacts with BSA, dipole–dipole interaction should change the T_2 values of nuclei of the ATP that interact closely with BSA. No proton chemical shifts of ATP were observed for BSA solutions (650 μM in 10 mM MES–NaOD, pH 5.4) containing ATP of 4.9 or 10.1 mM, and the measurement was done for these two solutions. Since P_b in Eq. (6) can be calculated using K_d ($= 1/K_{ATP}$) of ATP–BSA binding, T_{2f} and T_{2b} can be estimated from the two T_2 values obtained. The estimated values of T_{2f} and T_{2b} values for 2- and 8-H of ATP were estimated and are listed in Table 2. The values for protons attached to the sugar could not be determined because those signals were not adequately separated from the signal of water.

In Table 2, T_{2ATP}'s are those obtained in 10 mM ATP solutions buffered at pH 5.4 with 10 mM MES–NaOD. The estimated T_{2f} was very close to T_{2ATP}, suggesting that Eq. (6) is a good approximation because there are no chemical shifts. This result also suggests there is no non-specific interaction between ATP and BSA. The bottom row in the table represents the value of T_{2f}/T_{2b} that may give an estimation of the degree of interaction at each position.

3.5. Inhibition of various BSA-binding substrates on ATP-binding

BSA binds a variety of compounds, and several binding regions (sites) of BSA are reported. Sudlow et al. [20] classified three of these binding sites. Typical substrates of site-I are warfarin, phenprocoumon, iodipamide azapropazone, phenylbutazone and glibenclamide although a sub-class was proposed; site-II, diazepam, ibuprofen, bilirubin, flufenamic acid; site-III, digitoxin, digoxin. In addition to these, there are binding sites for long-chain fatty acids, medium-chain fatty acids, metal ions and anions.

Flufenamic acid (200 μM), a typical site-II reagent and digitoxin (200 μM), a typical site-III reagent had no effect on the ATP binding (data not shown) where 250 μM BSA in 25 mM MES–Tris buffer (pH 6.0) was used. Under this condition, their dissociation constants are reported to be 8.8 and 25 μM [1,2,21], respectively. (Flufenamic acid has a value of 8.8 μM for the binding to human serum albumin, HSA, and we assume that the value of BSA is not much different and that it might be less than 200 μM that was used for our experiment.) Hence, if ATP binds to the sites of these chemicals (i.e., site II or III), these concentrations are enough to observe the interference. Neither 400 μM of lauric acid nor the same amount of palmitic acid changed the Scatchard plot of ATP-binding (data not shown). The dissociation constant of fatty acid is on the order of 1 to 10 nM [1,2]. We concluded, therefore, that the ATP-binding site may not be site II, III or the site for fatty acids.

On the other hand, 200 μM of warfarin interfered with the ATP binding where 250 μM BSA in 25 mM MES–Tris (pH 6) was used, but the inhibitory effect was not appreciable even though about 10-fold more concentrated warfarin was used than its disso-

Table 2
T_{2f} and T_{2b} values for 2- and 8-proton of ATP

	Spin–spin relaxation time T_2 (ms)	
	8-H	2-H
T_{2b}	8.2	13
T_{2f}	65	246
T_{2ATP}	66	250
T_{2f}/T_{2b}	7.9	19

T_{2b} and T_{2f} stand for the spin–spin relaxation time for the bound and the free form of ATP calculated from Eq. (6) using P_b determined from ultrafiltration experiments.

ciation constant (20 μM) [1,2]. Since the plot of Eq. (6) did not yield the linear relationship (data not shown), warfarin is not a competitive inhibitor for ATP–BSA binding.

3.6. Competition with Cl^-

Fig. 6a shows the increase in K_d for ATP binding as an increase in NaCl concentration, where BSA of 350 μM in 25 mM MES–Tris at pH 7.4 was used and NaCl concentrations added were 10, 25 and 100 mM. Analysis in accordance with Kragh-Hansen [2] revealed competitive inhibition (see Fig. 6b) and the inhibition constant was determined to be 20 mM. ^{23}Na- and ^{35}Cl-NMR spectra were measured on addition of ATP to NaCl-equilibrated BSA solutions; we observed the narrowing of signal width of Cl^- while the width of Na^+ signal did not change (data not shown). These facts imply that ATP releases Cl from its binding site.

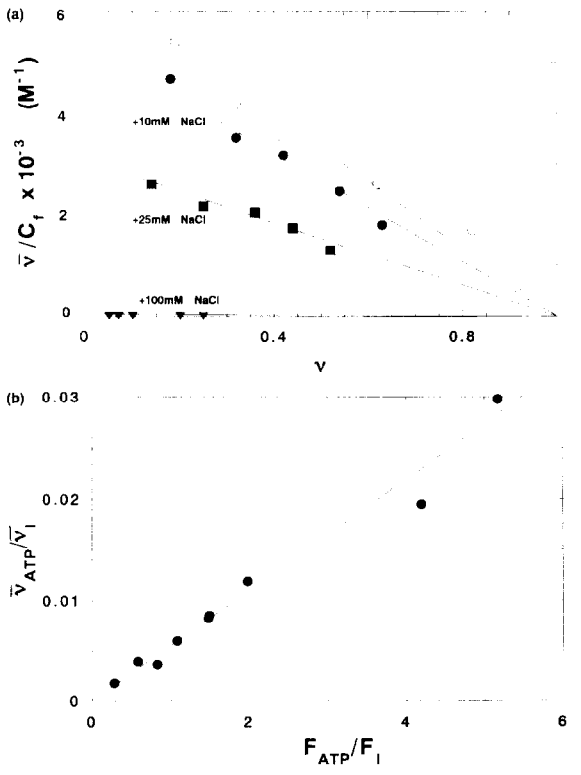


Fig. 6. (a) Scatchard plots of ATP–BSA binding in the presence of varying concentrations of NaCl. BSA of 350 μM dissolved in 25 mM MES–Tris at pH 7.4 was used, and temperature was 25°C. ○, no addition of NaCl; ●, 10 mM NaCl; ■, 25 mM NaCl; ▼, 100 mM NaCl. (b) NaCl is a competitive inhibitor for ATP–BSA binding. The BSA solution was 250 μM buffered with 25 mM MES–Tris at pH 7.4. Temperature was 25°C. It is noted that data obtained under various combinations of NaCl and ATP concentrations gave a single straight line.

3.7. Thermodynamic parameters

The temperature dependence of ATP binding yielded the following thermodynamic parameters: $\Delta H = -11.3$ kcal/mol, $\Delta S = -20.1$ cal/K/mol, and $\Delta G = -5.31$ kcal/mol at pH 7.4 and 25°C. Several thermodynamic data have been reported. For long fatty acids [1,22], $\Delta H = -17.7$ kcal/mol, $\Delta S = -2.5$ cal/K/mol, $\Delta G = -9.5$ kcal/mol; for warfarin [23] (but, to HSA), $\Delta H = -4.0$ kcal/mol, $\Delta S = 10.3$ cal/K/mol, $\Delta G = -7.2$ kcal/mol; for oxyphenylbutazone [23] (to HSA), $\Delta H = -2.5$ kcal/mol, $\Delta S = 15.2$ cal/K/mol, $\Delta G = -7.2$ kcal/mol; for L-tryptophan [24] (to HSA), $\Delta H = -2$ kcal/mol, $\Delta S = 15$ cal/K/mol, $\Delta G = -7$ kcal/mol. These data show that hydrophobic compounds give positive values of ΔS, as is evident by hydrophobic interaction [25]. The main driving force of the ATP binding is not the hydrophobic interaction.

3.8. Binding to non-mercaptoalbumin

The dissociation constant of ATP binding to non-mercaptalbumin was not changed, meaning that Cys-34 has nothing to do with the binding.

4. Discussion

These findings clearly show that ATP binds to BSA with a stoichiometry of 1:1. At pH 7.2, the dissociation constant was 120 μM, which is comparable to the value of 110 μM reported previously [11] using spin-labeled N^6–SL–ATP. Other spin probes such as C8–SL–ATP, C2′,C3′–SL–ATP and C2′,C3′–SL–AMP, on the other hand, gave a much lower value of 30 μM (for chemical structure, see Ref. [11]). Although the reason for the difference from our values for C8–SL–ATP, C2′,C3′–SL–ATP and C2′,C3′–SL–AMP is not clear, one possible

explanation is the difference in hydrophobicity between spin-probes and an isotope-labeled ATP used in our experiments. In fact, for a fluorescence ATP probe of 2′,3′-O-(2,4,6-trinitrocyclohexadienylidene) adenosine 5′-triphosphate, stronger binding was observed (data not shown) perhaps due to the hydrophobic interaction of the probe and BSA.

The dissociation constant of ATP binding decreased with decrease in pH. Although the reason is not clear at present, the following may be possible factors. First, albumin shows a pH-dependent structural change [1]: F-form at pH 3 to 5, N-form at pH 5 to 7, and B form at pH 7 to 9. Dissociation constant of warfarin is reportedly dependent on this structural change [26]. Second, the change in electric charges at or near the ATP-binding site. Third, the association of phosphate of ATP with H^+ may affect the binding.

Comparison of the K_1 values between GTP, UTP, phosphate and pyrophosphate (see Table 1) suggests the interaction of the base or sugar part of ATP. Actually, Table 2 shows the interaction of the base, especially 2-H of adenine, and Fig. 5 shows that the inhibition constant depends on the base. However, since adenine itself did not inhibit the binding, the interaction between the base and BSA does not seem a primary driving force for the ATP binding.

Table 1 shows that the order of strength as competitive inhibitors is (ATP) > ADP > AMP (also see Fig. 3) and pyrophosphate > phosphate (also see Fig. 4), suggesting that a series of negative charges is required for the binding site. The requirement of a series of negative charges on the substrates suggests the necessity of a series of positive charges on the binding site, although this might be a very simple hypothesis. The binding is pH-dependent and at around pH 6.5–7.0 the dissociation constant changes, which suggests the involvement of histidine residues of BSA, although there are other possibilities (see above). There are several amino acid sequences that fit the above hypothesis: Lys(239)–Val–His–Lys(242), Arg(335)–Arg–His(337), Lys(375)–Leu–Lys–His(378), Lys(533)–His–Lys–Pro–Lys(537). It is assumed [27] that the fragment of 239–242 is within a helix, that of 335–337 is the end of a helix, that of 375–378 is within a helix, and that of 533–537 is the end of a helix.

Contradicting the pioneer work by Bauer et al. [11] fatty acids had no effect on ATP-binding. For the strongest long-chain fatty acid, Lys-473 of BSA was shown to be most important, and for the second and third one, involvement of Lys-350 and Lys-116, respectively, was suggested [28]. Since our data revealed that fatty acids did not interfere at all, the ATP-binding site may not be a neighbor of these Lys-residues. Therefore, the above candidates of Arg(335)–Arg–His(337) and Lys(375)–Leu–Lys–His(378) might be ruled out.

We examined which compounds interfered with the ATP-binding and found that warfarin was a non-competitive inhibitor. In human serum albumin, the importance of Trp-214 on warfarin binding was attested by chemical modification [27,29]. Based on this and other observations, the region from Lys-199 to Glu-292 is proposed as the warfarin binding site. Since warfarin is a non-competitive inhibitor, the ATP-binding site might be close to this site.

We found that the dimer does not bind ATP, and this fact suggests that the ATP binding site might be near the amino-terminal, because dimer is presumably formed through a disulfide bond to Cys-34.

Our finding suggests strongly that the ATP-binding site is that for Cl^- binding. Three Cl^--binding sites have been recognized, the dissociation constants were reported to be 0.42, 5 and 143 mM [30] or 0.42, 10 and 303 mM [31], respectively; the numbers of the binding sites are 1, 8 and 18, respectively. The inhibition constant of Cl^- to the ATP binding was 20 mM; the value of the second binding site is the nearest to this value and then the site for ATP might be the second Cl^- binding site. Unfortunately, identification of the Cl^- binding site has not yet been made; the implication from ^{35}Cl-NMR was that this site might be a pair of basic residues such as Lys–Lys or Arg–Arg [32]. Identification of the ATP-binding site, therefore, will clarify the secondary Cl^--binding sites of BSA. We are now undertaking photoaffinity labeling with 8-azido-ATP to identify this site.

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