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lin-Benzo-ATP and -ADP: Versatile Fluorescent Probes for Spectroscopic and Biochemical Studies[1]

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lin-Benzo-adenine nucleotides can act not only as probes for fluorescence studies but also as structural active site probes for enzymes. To understand the basic properties of *lin*-benzo-ATP and -ADP, protolysis and Mg^{2+} and Ca^{2+} binding are investigated between pH 6.2 and pH 8.5 by spectrophotometric and spectrofluorometric titrations. Based on a reaction model, a set of equilibrium constants is determined which is consistent with all available experimental results. The p*K* values of the Mg^{2+} and Ca^{2+} complex of *lin*-benzo-ATP in the chosen medium are 4.6 and 4.1, respectively, and those for the corresponding diphosphate are 3.1 and 2.8, respectively. Fluorescence and absorption spectra are reported.

KEY WORDS: *lin*-Benzo-ATP; *lin*-benzo-ADP; dissociation constants; Mg^{2+} and Ca^{2+} binding; fluorescence spectra.

INTRODUCTION

Despite their wide biochemical significance, ATP and ADP exhibit no fluorescence properties suitable for spectroscopic studies. To overcome this disadvantage, several fluorophores have been linked chemically to both nucleotides. Other attempts involved the introduction of marked structural changes to the adenine moiety, for example, 1,N^6-etheno- or *lin*-benzo-ATP and -ADP, respectively. In the case of the *lin*-benzo derivative, the fluorescence of the adenine analogue is achieved by formally inserting two methene groups between the heterocycles of the base part [1–6], as shown in Fig. 1. This structural change leaves the chemical nature and the principal position of the hetero atoms unchanged but leads to a linear expansion of the base part. This expansion provides an interesting dimensional site probe for analyzing the geometric properties of the corresponding binding domain in adenine nucleotide-dependent enzymes.

In contrast to their promising spectroscopic and structural features, as initially demonstrated [2–6], the *lin*-benzoadenine nucleotides have not yet been widely used in biochemistry. Recent studies of *lin*-benzo-ATP as a substrate for the P-type ATPases such as gastric H^+, K^+-, renal Na^+,K^+-, and sarcoplasmatic Ca^{2+}-ATPase, have indicated that this ATP analogue is only weakly bound to these enzymes [7,8], which is likely to be a consequence of the structural base expansion. Thus *lin*-benzo-ATP acts in these circumstances as a suitable structural probe. In contrast to the enzymatic hydrolysis of ATP, the presence of K^+ does not increase the rate of *lin*-benzo-ATP hydrolysis catalyzed by H^+, K^+-ATPase [7] and Na^+,K^+-ATPase [8], which leads to unexpected mechanistic implications. Nevertheless, the hydrolysis of *lin*-benzo-ATP, for example, in the presence of Na^+,K^+-ATPase, can be monitored spectrofluorometrically in a direct manner [8].

For F-type ATPases *lin*-benzo-ADP and -ATP turned out to be excellent substrates and exhibited a high affinity binding [9–13]. All six nucleotide binding sites on mitochondrial F_1 could be occupied by *lin*-benzo-ADP; binding to the three catalytic as well as to the three noncatalytic sites resulted in strong quenching of the fluorescence of the analogue [9]. Mutational analysis and

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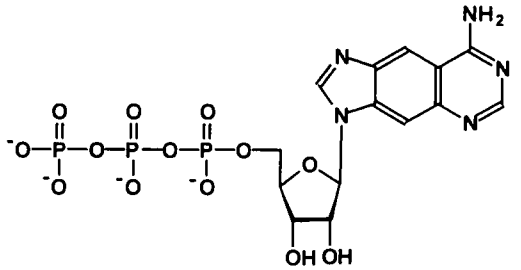

Fig. 1. Structure of *lin*-benzo-ATP (fully deprotonated state).

fluorescence studies with the *Escherichia coli* enzyme identified two tyrosine residues, one in either type of site, as responsible for the quenching effect [10,12]. These studies also gave an insight into the participation of individual amino acid residues in the formation of the binding sites and the role of these residues in ligand binding and substrate turnover. In addition, the spectral overlap between Trp fluorescence emission of the protein and *lin*-benzoadenine absorbance allowed the determination of distances via resonance energy transfer [11].

To interpret the interaction of *lin*-benzoadenosine nucleotides with enzymes, it is important to know the spectral properties of the different protolytic states and alkaline earth cation complexes as well as the corresponding equilibrium constants in a medium of similar ionic composition, which are reported here.

EXPERIMENTAL

The *lin*-benzo nucleotides were synthesized according to Refs 1, 2, and 4. The photometric concentration determinations are based on an extinction coefficient of $1.76 \times 10^4\ M^{-1}\ cm^{-1}$ at 260 nm (pH 7.5). All chemicals were of analytical grade. Absorption spectra were measured with a HP 8450 photometer and the fluorescence spectra with a Spex fluorolog 222 instrument employing thermostated cuvette holders. The titration medium consisted of 100 m*M* choline chloride as inert electrolyte to maintain approximately the same ionic strength during the titrations and 25 m*M* imidazole to maintain the pH. pH adjustment was done by adding concentrated solutions of HCl or recrystallized tetramethylammonium hydroxide. Cation titrations were performed by adding concentrated solutions of $MgCl_2$ (Suprapur Merck) and $CaCl_2$. During the titrations the cuvette solutions were continuously stirred. Errors of determined pK values were generally ±5%; for weak cation complexes, ±7%.

RESULTS AND DISCUSSION

As reported earlier [3,5], protolysis of the base moiety of *lin*-benzo-ATP and -ADP can be studied directly by carrying out spectrophotometric and spectrofluorometric pH titrations. The spectroscopic properties of the modified base are illustrated in Fig. 2. The experimentally determined pH dependencies can be analyzed on the basis of a single protonation or deprotonation step. The negative logarithm of the corresponding dissociation constants (pK_{23}) is significantly higher (cf. also Refs. 3 and 5) than those published for ATP and ADP [14–16].

Binding of Mg^{2+} or Ca^{2+} at a constant pH can also be followed directly by spectrophotometry or -fluorometry (cf. Fig. 2). Therefore, cation titrations have been carried out by employing these methods. The resulting titration curves correspond to a stoichiometric coefficient of 1. The apparent dissociation constant of cation binding (K_{app}) depends on the pH: pK_{app} increases with increasing pH (cf. Fig. 3). Furthermore, the spectral properties of the Mg^{2+} and Ca^{2+} complex depend on the pH, which indicates that the base part can exist as alkaline earth cation complex in the form of a protonated and deprotonated state. The corresponding dissociation constant (K_{45}) can be estimated by plotting the absorption or fluorescence intensities of the Mg^{2+} complex, as determined from the cation titrations at different constant pH values, versus pH, and on the basis of a fit procedure (cf. Table I). The pK_{45} value for the *lin*-benzo-ATP–Mg^{2+} complex was consistent with that determined from a pH titration performed with *lin*-benzo-ATP in the presence of 50 m*M* $MgCl_2$ in the medium mentioned above.

All experimental data obtained from pH and cation titrations (cf. Table I) are evaluated according to the reaction model shown in Fig. 4. The values for pK_{12}, corresponding to the deprotonation of the tri- or diphosphate residue, cannot be determined photometrically and are assumed to correspond to the values given for ATP and ADP [16]. Since the base part is not directly involved in alkaline earth cation coordination, this assumption seems to be justified. In addition, the pK_{45} values of the Ca^{2+} complexes have not been determined experimentally and are assumed to correspond to those found for the Mg^{2+} complexes. The values for pK_{24} and pK_{35} are obtained by fitting the theoretical function [17]

$$K_{app} = K_{24}\,\frac{1+10^{pH}/K_{12}(1+10^{pH}/K_{23})}{10^{pH}/K_{23}(1+10^{pH}/K_{45})} \qquad (1)$$

$$K_{35} = K_{24}\frac{K_{45}}{K_{23}}$$

to the experimental pK_{app} and related pH values. The

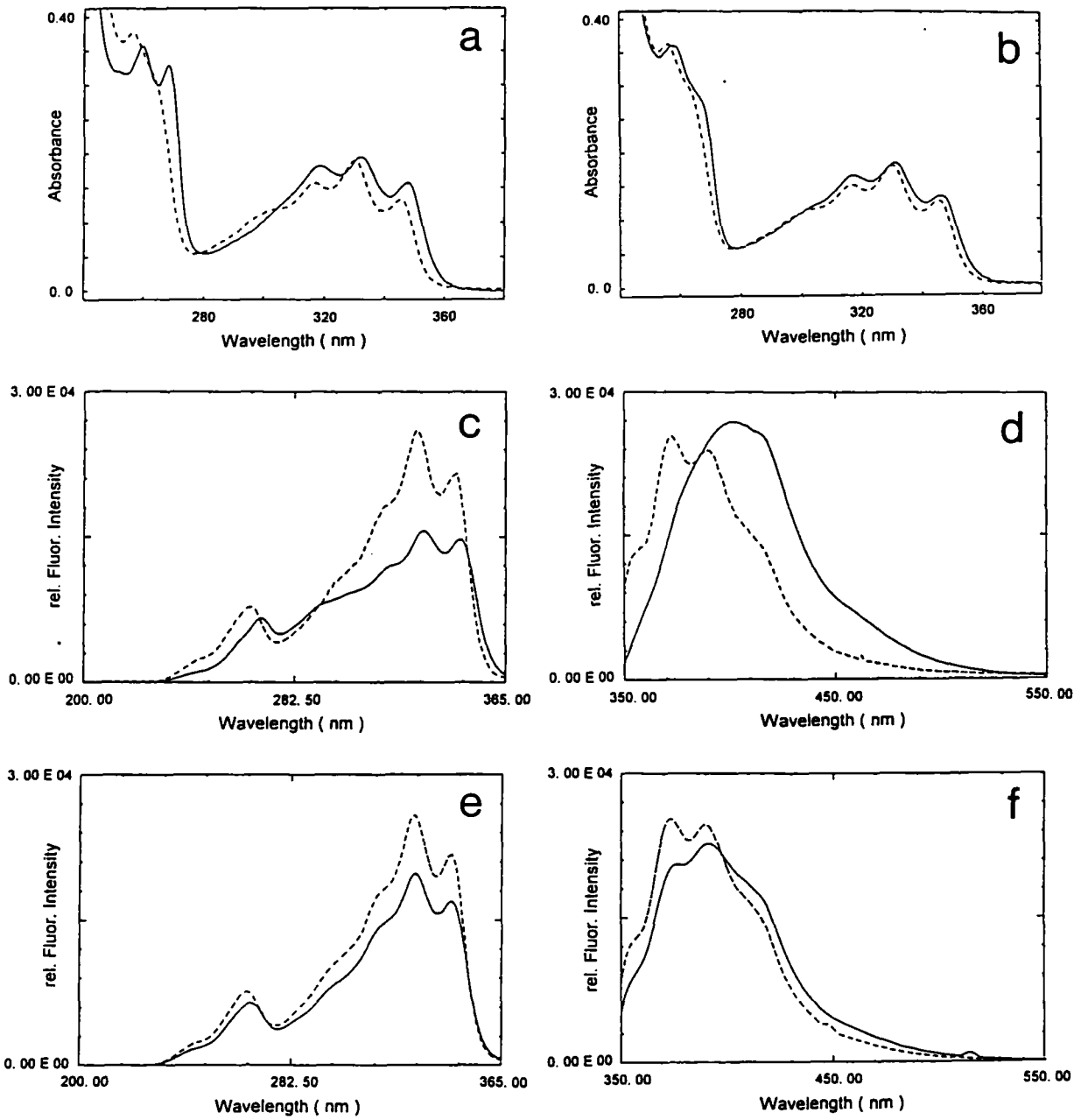

Fig. 2. Absorption and fluorescence spectra of *lin*-benzo-ADP at different pH values and of the Mg^{2+} complex in 25 m*M* imidazole/HCl containing 100 m*M* choline chloride (25°C). (a) Absorption spectra (conc., 20 μM; 1-cm path length) at pH 2.75 (——) and 9.40 (- - -) and (b) at pH 7.55 (——) as well as in the presence of 50 m*M* $MgCl_2$ (- - -). (c) Excitation spectrum (conc., 2.0 μM; emission at 372 nm) and (d) emission spectrum (excitation at 332 nm) at pH 2.75 (——) and pH 9.55 (- - -). (e) Excitation and (f) emission spectrum (experimental conditions as in c and d) at pH 7.55 (——) as well as in the presence of 50 m*M* $MgCl_2$ (- - -).

results are summarized in Table I. The pK_{24} values of the Mg^{2+} and Ca^{2+} complexes of *lin*-benzo-ATP and -ADP and the pK_{35} values of the Mg^{2+} complexes of both *lin*-benzo nucleotides are similar to those reported for the parent compounds [16]. This is consistent with the conception that Mg^{2+} and Ca^{2+} are preferentially coordinated to the phosphate residues and that only the charge of the base part affects the affinities. The pK_{35} values characterizing the complexes between Ca^{2+} and *lin*-benzo-ADP are considerably lower than the values given for the parent compound in [16] but close to those reported for ADP elsewhere [18,19]. It appears plausible

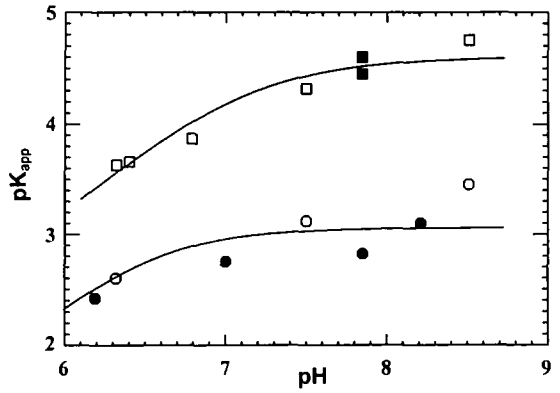

Fig. 3. Apparent dissociation constant of the Mg^{2+} complex of *lin*-benzo-ATP (squares) and -ADP (circles) in 25 m*M* imidazole/HCl at different pH values (25°C). The filled symbols refer to results obtained from spectrofluorometric and the open symbols from spectrophotometric titrations. The solid lines correspond to a fit according to Eq. (1). The resulting parameters are given in Table I.

Table I. Protolysis and Cation Binding of *lin*-Benzo-ATP and -ADP: Dissociation Constants (25°C)

Ligand	Cation	pK_{12}[a]	pK_{23}[b]	pK_{45}	pK_{24}[c]	pK_{35}[d]
lin-Benzo-ATP	H^+	6.51	7.2			
	Mg^{2+}			6.2[e]	3.7	4.6
	Ca^{2+}			6.2[f]	3.1	4.1
lin-Benzo-ADP	H^+	6.40	6.6			
	Mg^{2+}			6.2[e]	2.7	3.1
	Ca^{2+}			6.2[f]	2.4	2.3

[a] Assumed value as reported for ATP and ADP [16].
[b] Experimentally determined from pH titrations (error, ±5%).
[c] Result of fit procedure (error, ±7%).
[d] Result of fit procedure (error, ±7%).
[e] Experimentally determined and verified by fitting Eq. (1) (error, ±7%).
[f] Assumed to correspond to the value of the Mg^{2+} complex.

that the smaller cation interacts stronger with the tri- or diphosphate residue and, for electrostatic reasons, that the pK_{35} value for the triphosphate is higher than that for the diphosphate. The p*K* values of the Mg^{2+} complexes of *lin*-benzo-ATP and -ADP determined earlier at a high pH in a medium of different ionic composition by employing an indirect method [5] were slightly higher than the pK_{35} values reported here.

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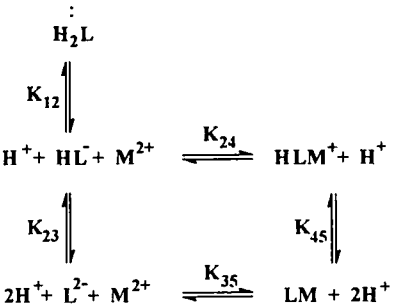

Fig. 4. Partial reaction scheme of alkaline earth cation binding (M^{2+}) to *lin*-benzo-ATP and -ADP. H_2L, HL^-, and L^{2-} represent the protolytic states of the nucleotides relevant for cation binding around neutral pH (the indicated charge does not correspond to the real one of the nucleotides). Assignments are given in the text.

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