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Isotachophoretic analysis of the pH dependent carrier properties of bovine serum albumin for rifamycin AF/013

The binding sites of bovine serum albumin for rifamycin AF/013 were examined by means of analytical capillary isotachophoresis. At pH 7.5–7.9, there was only one binding site on non-defatted bovine serum albumin, while under the same conditions up to 20 binding sites could be detected on fatty acid-free albumin. In both cases the interaction became nonspecific above pH 8.0, resulting in a large number of rifamycin AF/013 molecules bound to albumin. Co-binding with oleic acid *in vitro* resulted in competition with all but the first high-affinity binding site for rifamycin AF/013, corroborating the findings made with non-defatted albumin. Considering albumin as a model, the results might explain the conflicting reports about the number of binding sites of DNA-dependent RNA polymerases for rifamycin AF/013. Within a sharp pH-limit the highly specific binding becomes a nonspecific one. Moreover, the results suggest that the carrier function of bovine serum albumin might drastically change *in vivo*, depending on the pH of physiological fluids.

1 Introduction

Rifamycin AF/013, a derivative of rifamycin SV (Fig. 1), is an inhibitor of prokaryotic and eukaryotic DNA-dependent RNA polymerases [1–14]. Only the initiation step is inhibited, while elongation of RNA synthesis proceeds to completion in the presence of the inhibitor [1–3]. Based on studies with derivatives of rifamycin SV which act by binding to the enzyme [5], it was possible to characterize a series of events which precede the elongation step of transcription [6, 7].

Rifamycin AF/013 was found to be the derivative most suitable for the analysis of the eukaryotic transcription mechanism [1]. As rifamycin AF/013 is an inhibitor of the initiation step, it was used in titrations to assay the number of initiation sites for RNA polymerase B on different templates like DNA [8] or chromatin [9, 10]. It was reported that rifamycin AF/013 inhibits RNA polymerase as a function of enzyme concentration [1] and binds to albumin [11]. Rose *et al.* [12] observed that the extent, but not the mechanism, of inhibition by rifamycin AF/013 is altered by bovine serum albumin. This effect was most distinct with highly purified enzymes whereas the inhibition of less purified enzyme preparations was not substantially altered by further addition of exogenous proteins such as bovine serum albumin. However, less pure enzymes also required increased levels of rifamycin AF/013 to attain the same extent of inhibition [12]. Although several laboratories ([3, 11, 12]; A. Csordas and H. Grunicke, unpublished results), have observed the reduced inhibition by rifamycin AF/013 in the presence of albumin, the mechanism of the inhibitory effect of rifamycin AF/013 remained obscure.

Considering the complexity of the RNA polymerase reaction, one might anticipate only a limited number of rifamycin AF/013 binding sites on one RNA polymerase molecule, which consists of a large number of subunits [13, 14]. Riva *et al.* [11] investigated the binding properties of an RNA polymerase from a rifampicin-resistant mutant of *E. coli*. Although rifamycin AF/013 showed the same inhibitory effect on the wild-type enzyme as rifampicin, namely, interference only with the initiation step, its mechanism of action on the rifampicin-resistant enzyme was distinctly different. It was found that, at inhibitory concentrations, several hundred molecules of rifamycin AF/013 are bound to the enzyme molecule. Under these conditions, the binding of the rifampicin-resistant enzyme to the template was strongly reduced. Moreover, it was shown that bovine serum albumin protects the enzyme against the action of rifamycin AF/013. From this report one would have to conclude that, because of its hydrophobic nature, a large number of rifamycin AF/013 ligands interacts with proteins in a nonspecific manner.

The purpose of our investigation was to characterize the binding of rifamycin AF/013 to bovine serum albumin under different conditions and determine the number of high-affinity binding sites. The elucidation of the binding properties of albumin could serve as a model system for more complex protein molecules. Bovine serum albumin is available at a high grade of purity, its amino acid sequence is known [15] and domains of different ligand binding sites are characterized [16]. We have chosen the technique of analytical capillary isotachophoresis, which permits the analysis of compounds with

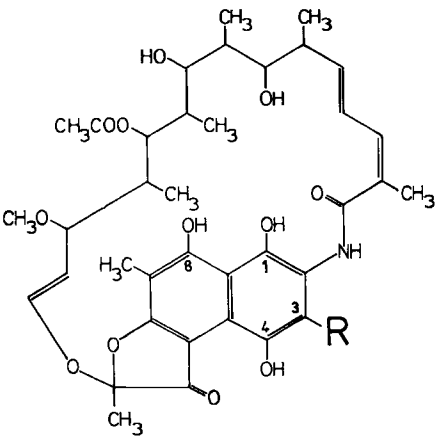

Figure 1. Structure of rifamycin SV and rifamycin AF/013.

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a minimum of handling in a Teflon capillary. This technique has already been applied to studies on binding of stable protein-ligand complexes [17–20]. Here we report about the distinctly different binding properties when comparing fatty acid-free and non-defatted albumin. It was also found that the pH value drastically affects the binding properties of rifamycin AF/013 to bovine serum albumin, which might have implications for the carrier properties of albumin and other proteins for rifamycin drugs in physiological fluids with elevated pH values.

2 Materials and methods

2.1 Materials

2.1.1 Bovine serum albumin

Non-defatted (A-7638) and fatty acid-free (A-0281) bovine serum albumins were purchased from Sigma (St. Louis, MO, USA). Before use they were extensively dialyzed against bidistilled water. The albumin solutions were adjusted to a concentration of 34 mg/mL (0.05 nmol/μL), using an ε_{mM} value of 45 and comparing with a non-dialyzed solution, the concentration of which was given by the weighed-in albumin powder. For purposes of calculation a molecular weight of 69 000 was used. Finally, the pH value was adjusted to 7.5–9.0, respectively, by addition of Tris(hydroxymethyl)-aminomethane.

2.1.2 Other chemicals

Rifamycin AF/013 was obtained from Gruppo Lepetit, Milano, Italy. Oleic acid (18:1, cis-9-octadecenoic acid, 0-0750, Sigma), barium hydroxide (Merck, Darmstadt, FRG) hydrochloric acid (suprapur, Merck), 2-amino-2-methyl-1-propanol (A-9879, Sigma), ε-amino-*n*-caproic acid (A-2504, Sigma), glycine (G-7126, Sigma), hydroxypropylmethylcellulose (H-7509, Sigma) and Tris(hydroxymethyl)aminomethane (No. 37190, Serva, Heidelberg, FRG) were obtained at the highest purity available.

2.1.3 Leading and terminating electrolytes

The leading electrolyte was 5 mM hydrochloric acid adjusted either to pH 7.5–8.3 by adding Tris(hydroxymethyl)-aminomethane or to pH 9.0 by adding 10 mM 2-amino-2-methyl-1-propanol; 0.2 % of hydroxypropylmethylcellulose (viscosity of 2 % aqueous solution approximately 4000 centipoises) was added to the leading electrolyte system in order to sharpen the zone boundaries by depressing electroendosmosis. The terminating electrolyte was either 10 mM glycine or 5 mM ε-amino-*n*-caproic acid adjusted to pH 9.20 and 10.50, respectively, with a freshly made $Ba(OH)_2$ solution. All electrolyte solutions were adjusted over a period of 2 days and controlled regularly thereafter.

Table 1. Composition of incubated samples of non-defatted bovine serum albumin and rifamycin AF/013

Sample[a)]	Non-defatted bovine serum albumin μL	Rifamycin AF/013 μL	Injected volume μL	Molar ratio of albumin to rifamycin AF/013
a	500	0	2	1:0
b	500	2.5	2	1:0.5
c	500	5	2	1:1
d	500	7.5	2	1:1.5
e	500	12.5	2	1:2.5
f	500	20	2	1:4

a) Refers to Fig. 3.

2.2 Methods

2.2.1 Incubation conditions of bovine serum albumin with ligands

The stock solutions of rifamycin AF/013 and oleic acid were prepared at fairly high concentrations so that their addition to the albumin solution would cause only small changes in volume. Increasing molar ratios of ligands were added to a constant amount of bovine serum albumin. For convenience and accuracy of sample preparation, 500 μL (0.5 nmol/μL) of the aqueous albumin solution were titrated with 50 nmol/μL (rifamycin AF/013 in dimethyl sulfoxide) or 500 nmol/μL (oleic acid in methanol) of the ligands (Table 1). After mixing, the final solutions were incubated at room temperature (25 °C) for at least 1 h prior to isotachophoretic analysis and were protected from light. No further components were added. The pH values of the solutions were controlled by means of a microelectrode and were found to remain stable within ± 0.03 pH. No formation of insoluble precipitates resulting from prolonged incubation was observed.

2.2.2 Isotachophoretic conditions

The principle of analytical isotachophoresis was the subject of several reviews [21–25]. The isotachophoretic analyses were carried out on an LKB 2127 Tachophor (LKB-Produkter AB, Bromma 1, Sweden) equipped with a 250 mm Teflon capillary of an internal diameter of 0.5 mm. By means of 5 μL Hamilton syringes (85NE, Hamilton Bonaduz AG, CH-7402 Bonaduz, Switzerland) the samples were injected through the inlet membrane into the leading electrolyte, which had the same pH value as the incubated samples. The separations were started at a current of 190–225 μA, which was gradually reduced to 25–50 μA shortly before the separated zones were detected by their conductivity, differential of conductivity and UV absorbance at 275 nm. All analyses were performed at a temperature of 25 °C and each run took about 20 min. The same results were obtained when the incubated samples were run in a longer capillary tube or at different detection currents, indicating that the isotachophoretic equilibrium was reached and no dissociation of the complex occurred.

2.2.3 Other methods

For ultrafiltration the disposable Amicon micropartition system Centrifree was used (Amicon Corp., Danvers/MA, USA). Absorption spectra at different pH values were scanned with a Beckman DU-8 spectrophotometer.

3 Results

3.1 Titration of high-affinity binding sites

For the isotachophoretic titration of high-affinity binding sites constant amounts of the protein were incubated with increasing molar amounts of the ligand, followed by injection into the Teflon capillary. At the beginning of an isotachophoretic run, the protein-ligand complex and the free ligand form separate zones, and during the run no exchange through the zone boundaries is possible. Thus, analyzing the samples with increasing molar ratios of ligand to protein, the saturation of protein with ligands can be monitored. If the protein is not saturated, only a signal of the protein-ligand complex is detected. After saturation of the protein molecule, an additional zone of the free ligand appears, marking the titration end-point, which can easily be determined owing to the high sensitivity of the detection system.

3.2 Isotachophoresis of the individual components

The profiles of conductivity and differential of conductivity signals obtained by analyzing the individual components of the incubation mixtures confirm by the sharply demarcated boundaries the homogeneity of non-defatted albumin, fatty acid-free albumin and rifamycin AF/013 (Fig. 2). The small UV-absorbing spikes were also observed when analyzing a blank. Rifamycin AF/013 is conspicuous by a higher UV-absorption than albumin.

3.3 Binding properties of non-defatted albumin

On titration of the binding sites of non-defatted bovine serum albumin for rifamycin AF/013 at a pH value of 7.90 (Fig. 3; see also Table 1) only at a molar ratio of 1:1 does the signal for free rifamycin AF/013 become clearly visible as an additional new zone in the UV recording, indicating that the albumin molecule is saturated. Further increases in the molar amount of rifamycin AF/013 result in a broadening of the zone of the free ligand (which is a measure of the molar amount) while the zone height (a function of the molar extinction coefficient) remains unchanged. Increasing the concentration of rifamycin AF/013 at equilibrium does not result in further binding,

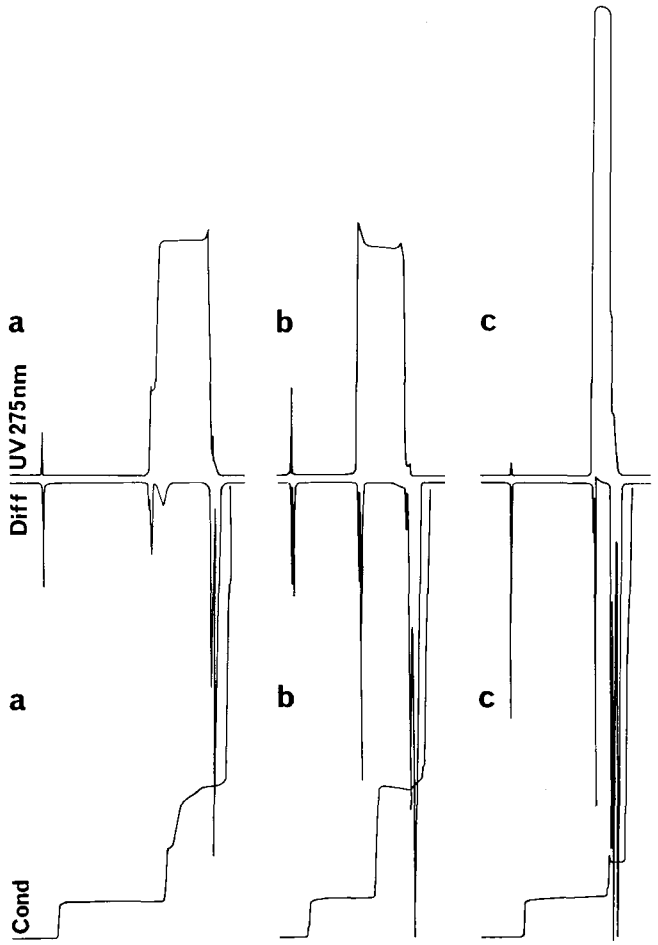

Figure 2. Isotachophoresis of the pure proteins and ligand. (a) Non-defatted bovine serum albumin, (b) fatty acid-free bovine serum albumin, and (c) rifamycin AF/013. UV-absorbance at 275 nm, conductivity and differential of conductivity were measured simultaneously. Temperature: 25 °C; pH value of the leading electrolyte: 7.80; detection current: 50 μA; chart speed: 0.2 mm/s; sample volumes: (a) 2 μL; (b) 2.5 μL and (c) 5 μL.

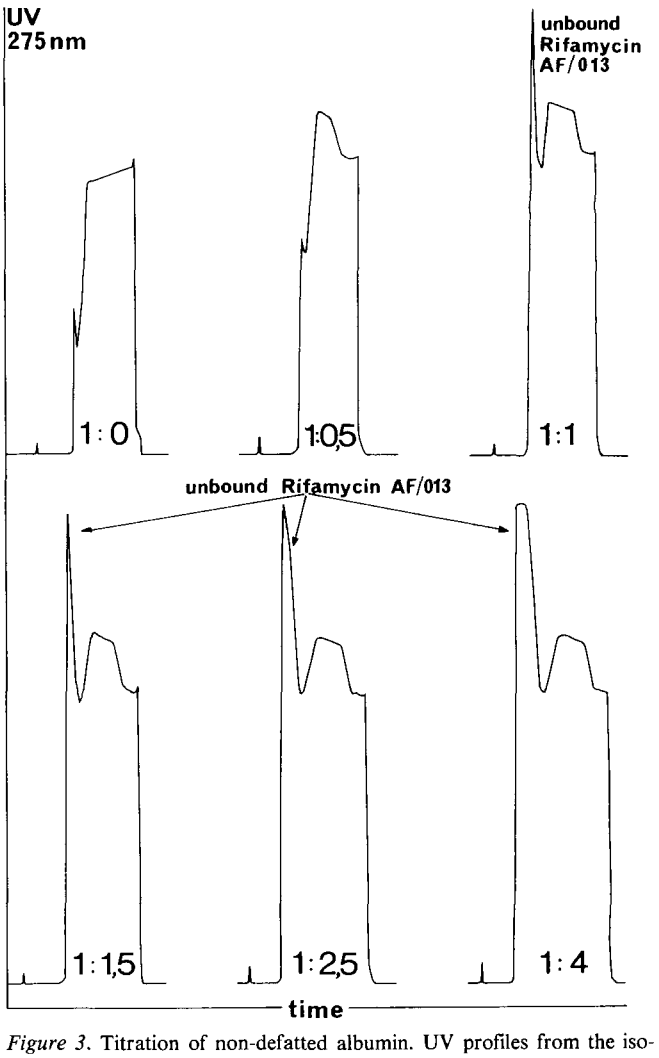

Figure 3. Titration of non-defatted albumin. UV profiles from the isotachophoretic analysis of incubation mixtures containing various ratios of non-defatted bovine serum albumin to rifamycin AF/013: (a) 1:0, (b) 1:0.5, (c) 1:1, (d) 1:1.5, (e) 1:2.5, and (f) 1:4. Temperture: 25 °C; pH value of the leading electrolyte: 7.90; detection current: 40 μA; chart speed: 0.2 mm/s; sample volumes: 2 μL. For identification of the composition of incubated samples see Table 1.

proving that at pH 7.9 there is only one high-affinity binding site detectable on non-defatted bovine serum albumin (Fig. 4). Subsequent titrations at pH values ranging from 7.5–8.0 also revealed only one binding site.

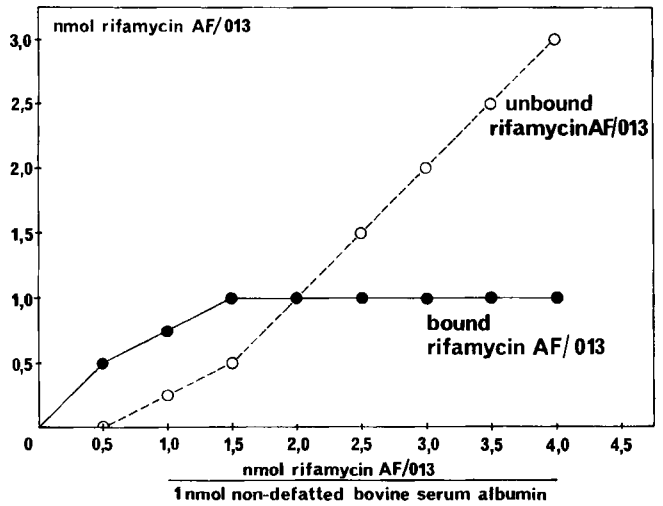

Figure 4. The titration of non-defatted albumin (*cf.* Fig. 3) plotted as a diagram showing the amount of bound and unbound rifamycin AF/013 in the individual incubation mixtures.

3.4 Binding properties of fatty acid-free albumin

The UV profiles of the titration of fatty acid-free bovine serum albumin with rifamycin AF/013 at pH 7.80 (see also Table 2) reveal that no free rifamycin AF/013 can be detected in incubation mixtures containing less than 20 nmol of rifamycin AF/013 per nmol of fatty acid-free bovine serum albumin (Fig. 5). However, when a molar ratio of 20 is reached, a sharply demarcated zone appears and on further increasing the molar concentration of rifamycin AF/013 the length of this additional zone of free ligand continues to augment. Identical results were obtained at pH 7.90. Comparing the results of Fig. 3 and Fig. 5, the different migration behavior of unbound rifamycin AF/013 becomes evident. On titration of non-defatted albumin the free ligand migrates ahead of the albumin-ligand complex, while for the titration end-point of fatty acid-free albumin a signal behind the complex is observed. Free rifamycin AF/013 was identified by the zone height of the UV-absorbance and conductivity signals, two characteristic properties for a given compound. These values were always identical for rifamycin AF/013, regardless of the

Table 2. Composition of incubated samples of fatty-acid-free bovine serum albumin and rifamycin AF/013

Sample[a)]	Fatty-acid-free bovine serum albumin µL	Rifamycin AF/013 µL	Injected volume µL	Molar ratio of albumin to rifamycin AF/013
a	500	0	2.5	1 : 0
b	500	20	2.5	1 : 4
c	500	75	2.5	1 : 15
d	500	95	2.5	1 : 19
e	500	100	2.5	1 : 20
f	500	110	2.5	1 : 22

a) Refers to Fig. 5.

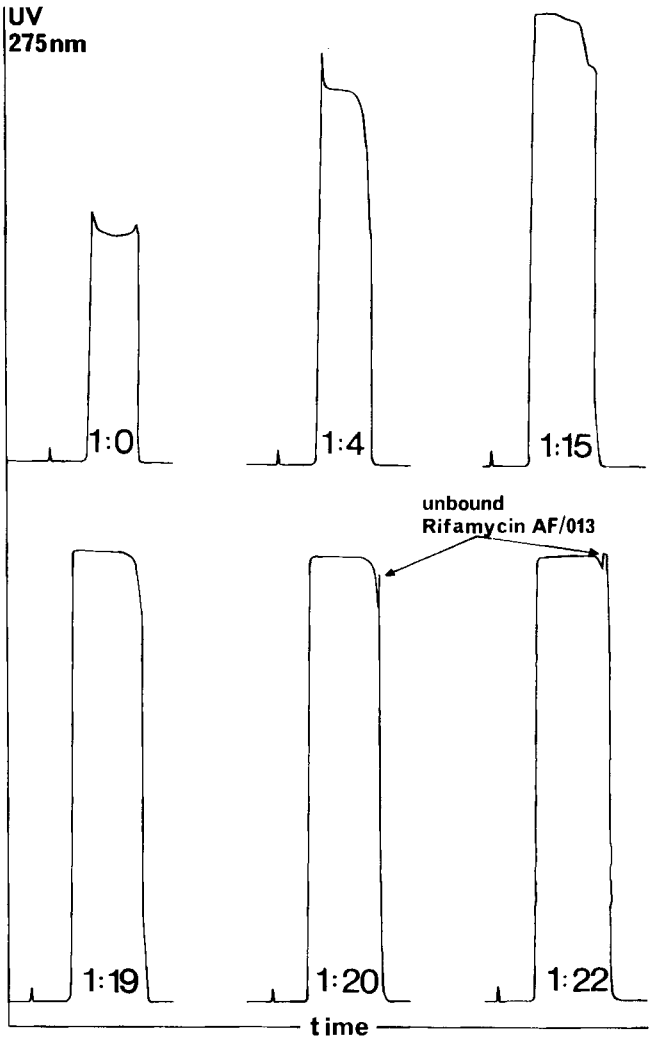

Figure 5. Titration of fatty acid-free bovine serum albumin with rifamycin AF/013. UV profiles from the isotachophoretic analysis of incubation mixtures containing various ratios of fatty acid-free bovine serum albumin to rifamycin AF/013: (a) 1:0, (b) 1:4, (c) 1:15, (d) 1:19, (e) 1:20, and (f) 1:22. Temperature: 25 °C; pH value of the leading electrolyte: 7.80; detection current: 50 µA; chart speed: 0.2 mm/s; sample volumes: 2.5 µL. For identification of the composition of incubated samples see Table 2.

different relative mobilities of the free ligand and the protein-ligand complex. For both albumins the identity of the signal for free rifamycin AF/013 was confirmed by internal standards. Injection of defined concentrations of free rifamycin AF/013 resulted in a proportional broadening only of the peaks identified as free ligand. The most likely explanation for the different relative mobilities of the albumin-ligand complex and free ligand, comparing defatted and non-defatted albumin, is the difference in the number of bound ligand molecules in the saturated complex. In the titration of fatty acid-free bovine serum albumin binding of up to twenty rifamycin AF/013 molecules with fully dissociated phenolic groups may be assumed with resultant increase in the net electrophoretic mobility, until the complex has an effective mobility higher than that of the free ligand. The differential binding properties of rifamycin AF/013 to non-defatted and fatty acid-free bovine serum albumin are illustrated in Fig. 6. For a strongly UV-absorbing material like rifamycin AF/013, the titration can be traced also by an increase in peak heights of

UV-absorbance of the albumin-rifamycin AF/013 complex, accompanying higher molar ratios of bound ligand even before the signal for free ligand appears (Fig. 5). This affords an advantage when analyzing protein-rifamycin complexes because other ligands, such as sodium lauryl sulfate or fatty acids, do not cause an increase in UV absorbance on binding to albumin.

3.5 pH Dependence of the albumin-rifamycin AF/013 interaction

At pH > 8.1 a drastic change in the binding affinity of non-defatted and also of fatty acid-free albumin was observed. The number of binding sites rose above 30 to a non-measureable extent.

3.6 Assay of free rifamycin AF/013

The molar amount of free rifamycin AF/013 was calculated for all previously described titrations from calibration curves (Fig. 7). Linearity was observed at concentrations ranging from 0.25 to 8mM of rifamycin AF/013. In the case of saturated complexes, the amount of free ligand could also be assessed by the addition of internal standards. In repeated determinations under identical conditions but with different volumes (1–3 μL) and molar ratios of albumin to rifamycin AF/013, an intra-assay and inter-assay precision of ±1.3 % and ±2 %, respectively, was obtained, the main errors resulting from the preparation (weighing-in and dissolving) of the solutions, the injection of the samples and the measurements of the zone length.

3.7 Co-binding of oleic acid

The distinctly different binding properties of non-defatted and fatty acid-free bovine serum albumin (Fig. 6) raise the question whether rifamycin AF/013 and fatty acids compete for

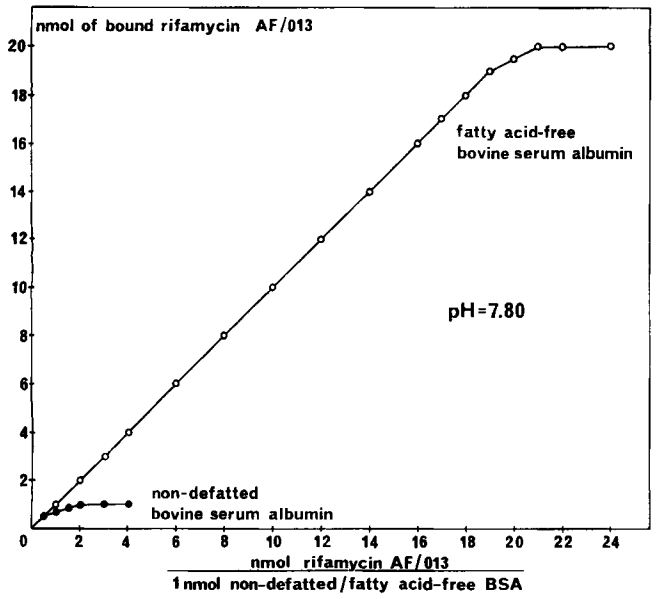

Figure 6. Lipid-dependent binding properties. Differential binding characteristics of non-defatted and fatty acid-free bovine serum albumin for rifamycin AF/013 at pH 7.80. Identical binding properties are exhibited by albumin in the pH range 7.5–7.9.

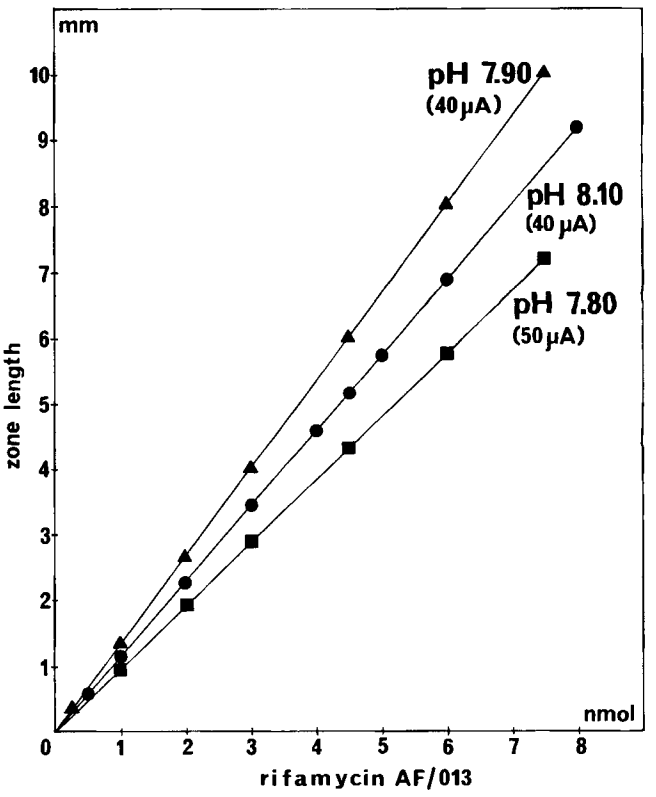

Figure 7. Assay of the free ligand. Calibration curves showing the linear relationship between nmol of rifamycin AF/013 injected and the corresponding zone length in mm, measured from the differential of conductivity profile at different pH values and detection currents.

Table 3. Composition of incubated samples of fatty-acid-free bovine serum albumin, rifamycin AF/013 and oleic acid

Sample[a)]	Fatty acid-free bovine serum albumin μL	Rifamycin AF/013 μL	oleic acid	Injected volume μL	Molar ratio of albumin to rifamycin AF/013 and oleic acid
a	500	95	0	2.5	1 : 19 : 0
b	500	95	2.5	2.5	1 : 19 : 5
c	500	95	5	2.5	1 : 19 : 10
d	500	5	0	2.5	1 : 1 : 0
e	500	5	25	2.5	1 : 1 : 50

a) Refers to Fig. 8.

the same binding sites. As shown in Fig. 8 (see also Table 3), oleic acid partially displaced rifamycin AF/013. However, no displacement from the first high-affinity binding site could be achieved even at concentrations far exceeding any physiological level (molar ratio of oleic acid to albumin 50:1, Fig. 8). The *in vitro* experiments corroborate previous observations on albumin loaded with lipids *in vivo* (non-defatted albumin). Thus, there is only one binding site with higher affinity for rifamycin AF/013 and up to 19 more which show a higher affinity for the *in vivo* lipids, and from which *in vitro* rifamycin AF/013 can be displaced by oleic acid. These possibly represent specific binding sites for free fatty acids.

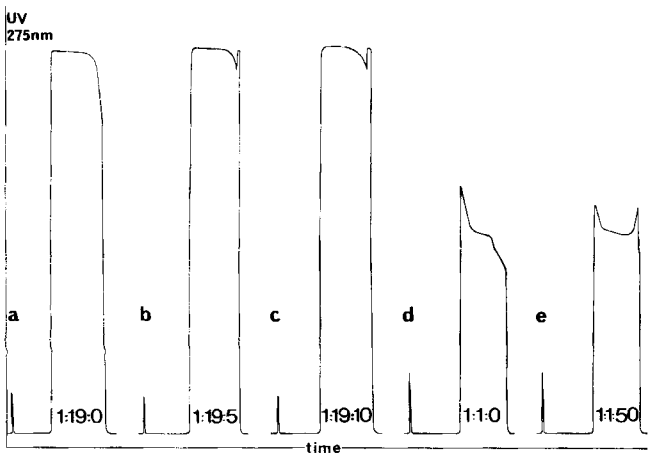

Figure 8. Co-binding of oleic acid and rifamycin AF/013 to defatted bovine albumin. UV profiles from the isotachophoretic analysis of incubation mixtures containing various ratios of fatty acid-free bovine serum albumin to rifamycin AF/013 and oleic acid: (a) 1:19:0, (b) 1:19:5, (c) 1:19:10, (d) 1:1:0, and (e) 1:1:50. Temperature: 25 °C; pH value of the leading electrolyte: 7.80; detection current: 50 μA. Chart speed: 0.2 mm/s; sample volumes: 2.5 μL. For identification of the composition of the incubated samples see Table 3.

4 Discussion

4.1 pH Dependence of ligand binding

Depending on pH and lipid content, there is either a specific or a nonspecific interaction between rifamycin AF/013 and bovine serum albumin. Non-defatted albumin shows only one binding site for rifamycin AF/013 in the pH range from 7.5–8.0. As a result of defatting, 19 additional binding sites are observed, occupied by oleic acid even after prior saturation with rifamycin AF/013. These additional sites represent most likely binding sites for free fatty acids. It is noteworthy that defatted and non-defatted bovine serum albumin did not exhibit any marked difference in their binding properties to bilirubin [20].

At pH $\geq$ 8.0 there is a sharp transition to a third type of interaction, namely nonspecific binding by both fatty acid-free and non-defatted bovine serum albumin. The molar ratio of bound rifamycin AF/013 to albumin increases enormously and cannot be determined any more by capillary isotachophoresis. This nonspecific type of binding is reminiscent of the observation of Riva *et al.* [11] who found that a rifampicin-resistant RNA polymerase binds up to several hundred molecules of rifamycin AF/013. The pH dependence of binding might also explain the high antibacterial activity of rifamycin drugs in the alkaline milieu of the infected prostatic fluid.

The drastic pH-dependent change of the binding properties of bovine serum albumin for rifamycin AF/013 may result either from a pH-dependent alteration of the ligand or a conformational change of the albumin molecule. The pH-dependent change of the binding properties could occur at a pH corresponding to one of the pK_a-values of the phenolic groups in rifamycin AF/013 (Fig. 1). However, when absorption spectra in the visible and in the UV-range (200 nm–600 nm) at pH 7–10 were compared, no shift of any peak to another wavelength could be observed. Based on data obtained with different rifamycins, the first pK_a-value of rifamycin AF/013 should be lower than 4. The derivatives of rifamycin SV have this unusual low value of pK_a, between 2 and 3, due to the formation of a complex system of hydrogen bonding between the phenolic -OH groups on carbon 8, on carbon 1 and the amide carbonyl group with resultant easy dissociation of one of the protons and a stronger binding of the other. Analysis of the UV-spectra of rifamycin SV [26], at pH $\geq$ 7 has shown the system of phenolic groups to be completely dissociated. Therefore we favor the alternative explanation for the pH-dependent change in the binding properties namely, a pH-dependent conformational change of the albumin molecule which is known to occur for instance after binding of low concentrations of fatty acids [27]. The sharp transition from specific to nonspecific binding might explain the conflicting reports in the literature about a specific type of inhibition of the initiation step of RNA polymerases on the one side [1–3], and a nonspecific type of binding on the other hand [11]. Usually, the assay of DNA-dependent RNA polymerases is performed at pH 7.9–8.1. This is exactly the range where, at least as far as bovine serum albumin is concerned, the change to nonspecific binding occurs. It remains to be investigated whether RNA polymerases exhibit a similar pH dependence of binding.

4.2 Physiological implications

For the characterization of the physiological role of albumin it is important to know to what extent different ligands like metabolites and drugs interfere with its transport capacity. For this reason we compared the binding properties of non-defatted and fatty acid-free bovine serum albumin. A remarkable number of binding sites is exposed as a result of the defatting procedure (Figs. 5 and 6). Again, two interpretations can be considered: (i) Binding sites occupied by fatty acids or lipids under physiological conditions may become available after lipid extraction. (ii) The removal of lipids may result in a conformational change of the albumin molecule, disclosing new binding sites with a high degree of specificity. In an *in vitro* approach, increasing amounts of oleic acid were added to a saturated complex of fatty acid-free albumin with rifamycin AF/013. Only one binding site of albumin was resistant to the *in vitro* competition with oleic acid, confirming previous results with albumin loaded with serum lipids *in vivo* (non-defatted albumin), which demonstrated only one binding site for rifamycin AF/013.

4.3 Potential of analytical capillary isotachophoresis

This report demonstrates that analytical isotachophoresis in a capillary tube, due to the distinct separation of saturated protein-ligand complexes and free ligands, provides a powerful

and convenient tool for studying the competitive or synergistic influences of drugs and metabolites, such as long chain fatty acids, on the transport capacity of albumin for a specific ligand. Thus, a hierarchy of binding affinities can be established. However, as with gel filtration or sucrose gradients, only stable protein-ligand complexes can be examined. The use of an inert Teflon capillary and the short time required for an isotachophoretic run (10–20 min) are a great advantage of capillary isotachophoresis for the analysis of substances which tend to be adsorbed to glass and other routine laboratory materials or are light-sensitive and unstable. Our attempts, for instance, to analyze binding affinities by means of ultrafiltration failed because rifamycin AF/013 and bilirubin were bound by the filters. The results presented in this paper show that isotachophoresis can be applied for the analysis of stable protein-ligand complexes. Our studies revealed that the binding of rifamycin AF/013 to bovine serum albumin can be highly specific with a single binding site, but only under the appropriate conditions. This might be helpful for studies with RNA polymerase B when rifamycin AF/013 is used as an inhibitor.

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