Conformational changes in the bilirubin–human serum albumin complex at extreme alkaline pH

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Light-absorption, c.d. and fluorescence of the bilirubin–albumin complex were investigated at extreme alkaline pH. Above pH 11.1 albumin binds the bilirubin molecule, twisted oppositely to the configuration at more neutral pH. On the basis of light-absorption it is shown that two alkaline transitions occur. The first alkaline transition takes place at pH between 11.3 and 11.8, co-operatively dissociating at least six protons. The second alkaline transition takes place at pH between 11.8 and 12.0. It probably implies a reversible unfolding of the albumin molecule, increasing the distance between tryptophan-214 and bilirubin, and partly exposing the liganded bilirubin to the solvent.

INTRODUCTION

It is well known that albumin undergoes a number of pH-dependent conformational transitions (Foster, 1977). At neutral pH the normal or N form exists. Decreasing the pH below 4 results in the F form, and at pH around 2–3.5 the so-called acid expansion occurs. On the alkaline side above pH 8 the B form is found, slowly ‘aging’ to the A form. Little interest has been focused on the extreme alkaline pH beyond 11, where tyrosine residues are deprotonated, resulting in a co-operative transformation of albumin (Decker & Foster, 1967; Steinhardt & Stocker, 1973). Studying the bilirubin–albumin complex, Brodersen (1979) noted a marked change in light-absorption spectra when pH was varied around 11.6. At pH 12 the spectrum was blue-shifted, becoming much like that of the unbound bilirubin. He proposed that albumin had undergone a conformational change (B–C transformation), cleaving the bilirubin–albumin complex, together with a co-operative deprotonation involving ten tyrosine residues.

In the present paper we have made a more detailed analysis of the bilirubin–albumin complex between pH 11 and 12. The titration experiment made by Brodersen (1979) has been repeated with greater precision, and by using fluorescence and c.d. measurements it is shown that the spectral shift is caused by conformational changes in the bilirubin–albumin complex and not by scission of bilirubin from the complex.

EXPERIMENTAL

Materials

Human serum albumin was obtained from AB Kabi, Stockholm, Sweden. It was defatted with charcoal in acid solution in accordance with Chen (1967a) and freeze-dried. Stock solutions of albumin were made by weighing of the dry sample, the M_r being taken as 66000. The protein was dissolved in 0.1 M-NaCl and the pH was adjusted to about 9 with 1 M-NaOH.

Bilirubin was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and purified as described by

McDonagh & Assisi (1972). A 4 mg portion of bilirubin was dissolved in 200 μ l of 0.5 M-NaOH. On addition of 5 ml of water and finally 75 μ l of 1 M-HCl, the concentration was 1.3 mM. A volume of this solution was immediately mixed with the albumin, or an alkaline solution of 0.1 M-NaCl for experiments without albumin. In the latter case these were run within an hour. The bilirubin solutions were handled under orange light.

Methods

Acidimetric titration. A 15 ml portion of a 60 μ M-albumin/30 μ M-bilirubin solution in 0.1 M-NaCl was titrated with 2 M-NaOH at 25.0 °C with the use of an Agla micrometer syringe from Wellcome Reagents (Dartford, Kent, U.K.) allowing a minimum sample delivery of 0.2 μ l. The syringe was protected from temperature fluctuations, and the entire titration set-up was confined in a soft-iron plate cabinet to prevent electric interference. Readings of pH were made to three decimal places with a model 26 pH-meter, with a G2040B glass electrode and a K102-K reference calomel cell connected to a K102-B salt bridge from Radiometer (Copenhagen, Denmark). The reference electrode was kept at 25.0 °C during a period of at least 24 h before and during titration. Within 15 min the titration curve was found to be wholly reversible up to pH 12.1.

Spectrophotometric titration. This was done on a 60 μ M-albumin/30 μ M-bilirubin solution. Light-absorption was observed in an ACTA mark VI spectrophotometer from Beckman Instruments (Fullerton, CA, U.S.A.) with cell holder thermostatically maintained at 25.0 °C. The titration curve was used for pH determinations. Maximum increase of volume at pH 12 was less than 1%, and absorption was corrected for this.

The stability of albumin at pH 12 was checked by running u.v.-light-absorption spectra at intervals after preparation of the solution. It was found to be stable for at least 15 min. The visible-light-absorption spectra of the bilirubin–albumin complex were stable for at least 30 min at pH below approx. 11.8. Between pH 11.8 and 12.1 a steady decrease of absorbance was seen (approx.

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0.2%/min). This was probably caused by oxidation of bilirubin without irreversible damage to the albumin molecule. The reversibility of bilirubin light-absorption spectra was checked by keeping exposure times at high pH values at a minimum. In this way reproducible spectra similar to those pictured in Fig. 1 could be obtained.

C.d. C.d. was measured in a model II dichrograph CD 185 from Roussel-Jouan (St. Germain, Paris, France). Determinations were done at 26 ± 1 °C in a 10 mm cell with 60 μM -albumin/30 μM -bilirubin solutions at different pH values, each time freshly prepared from a stock solution at pH 9. The dichrograph was calibrated with epiandrosterone in dioxan, and the results are presented as the molar c.d. absorption coefficient $\Delta\epsilon$, based on the total bilirubin concentration. Albumin gives no measurable contribution to the c.d. between 340 nm and 550 nm. By preparing the solutions as described above, they were stable for at least 45 min (pH 11.6). Reversibility was checked with a solution of bilirubin–albumin complex kept at pH 11.9 for approx. 15 min, and the c.d. at pH 11.4 could then be reproduced quantitatively, indicating that no major damage occurred to albumin or bilirubin in this time interval.

Fluorescence experiments. These were carried out on an Aminco–Bowman spectrophotofluorimeter (American Instrument Co., Silver Spring, MD, U.S.A.) with cell holder thermostatically maintained at 25.0 °C. The xenon lamp was used with a magnetic arch-stabilizer. Stability was checked with quinine sulphate (1 $\mu\text{g}/\text{ml}$ of 0.05 M- H_2SO_4) and found to be within 3%, which was sufficient for our purpose. A Philips PM 8120/01 flat-bed recorder was used for recording emission spectra. Fluorescence spectra of 4 μM -albumin with and without 2 μM -bilirubin were made at pH 11 and pH 12. The pH was adjusted with 1 M-NaOH at pH 11 and 4 M-NaOH at pH 12. Dilution was below 0.5%. Excitation was at 290 nm, and emission spectra uncorrected for variations in phototube response with wavelength were recorded. The absorbance was below 0.2. With the recorder connected to a time base the fluorescence of 4 μM -bilirubin with and without 8 μM -albumin was measured as a function of time at 520 nm, when excited at 450 nm. The pH was adjusted with 1 M-NaOH to the desired value from a stock solution at pH 9. Dilution was below 3%. The initial fluorescence was found by retropolation to zero time.

Gel electrophoresis. Samples of albumin exposed to pH 12 for 15 min were examined by gel electrophoresis in accordance with Laemmli (1970), with the modifications described by Andersen *et al.* (1985). Experiments with 1 mg/ml and 0.1 mg/ml albumin solutions were run, in the latter case both with and without reduction with 2-mercaptoethanol. All samples migrated similarly to a standard albumin solution kept at pH 9. No increase in dimer content and no denatured components were found, as seen with albumin kept above pH 10 for 96 h (Nikkel & Foster, 1971). No cleavage products were seen.

RESULTS

Light-absorption of bilirubin

Absorption spectra of the bilirubin–albumin complex

Fig. 1. Light-absorption spectra of the bilirubin–albumin complex in alkaline solutions

The pH was varied as indicated in solutions containing 30 μM -bilirubin without albumin (----) and with 60 μM -albumin (—) in 0.1 M-NaCl. The temperature was 25 °C.

at various pH values are shown in Fig. 1. Raising the pH from 11.3 to 12.1 gives a marked blue-shift of the spectrum. In the interval pH 11.3–11.8 an isosbestic point is obtained at 445 nm, but in the interval pH 11.8–12.1 this isosbestic point vanishes and is replaced by two new ones at 441 and 502 nm. When the absorbance is measured at 502 nm (i.e. the isosbestic point in the interval pH 11.8–12.1) as a function of pH, plateaus are found at pH 11.2–11.3 and pH 11.8–12.1 (Fig. 2). At 480 nm, on the other hand, the plateau at pH 11.8–12.1 is replaced by a plateau at pH 12.0–12.1 (curve not shown). The simplest explanation, then, is that two conformational transitions occur, and three albumin conformations are found. One exists at pH 11.2–11.3, a second around pH 11.8, and a third at pH 12.0–12.1.

The first alkaline transition (at pH 11.3–11.8) gives a marked blue-shift of the bilirubin spectrum, probably due to changes of exciton splitting between the two bilirubin chromophores (see under 'C.d. of bilirubin' below). The transition could be observed by the absorbance at 502 nm (Fig. 2), plotted in a Hill plot (Fig. 3). The midpoint of the titration was pH 11.45, and the Hill coefficient in two experiments was 6.1 and 6.6. This indicates that at least six protons dissociate co-operatively from the bilirubin–albumin complex in the first alkaline transition.

The second alkaline transition (at pH 11.8–12.0) mainly gives a slight blue-shift of the bilirubin spectrum. It could be explained by scission of bilirubin from the complex (Fig. 1), but this is contradicted by the fluorescence experiments (see below). A blue-shift is expected when a $\pi \rightarrow \pi^*$ excitation is perturbed by making the surroundings more polar (Donovan, 1969). At pH 10 both bilirubin chromophores are buried in the albumin molecule, and shielded from contact with the solvent (Jacobsen & Brodersen, 1983). A possible explanation of the spectral change would then be a partial exposure of the bilirubin chromophores to the more polar solvent when pH is raised from 11.8 to 12.1.

Fig. 2. Spectrophotometric titration curve for the bilirubin-albumin complex

The absorbance was measured at 502 nm in a solution containing $30\ \mu\text{M}$ -bilirubin and $60\ \mu\text{M}$ -albumin in $0.1\ \text{M}$ -NaCl. The pH was raised by adding known amounts of $2\ \text{M}$ -NaOH. The two different symbols represent results from two different experiments. The temperature was $25\ ^\circ\text{C}$.

Fig. 3. Hill plot for the association of protons to the bilirubin-albumin complex

The data in Fig. 2 were transformed into this Hill plot. From two different experiments the Hill coefficient (h) was 6.6 (○) and 6.1 (□) respectively. The same symbol in Figs. 2 and 3 represents the same experiment.

Fig. 4. C.d. of the bilirubin-albumin complex at various pH values

The c.d. at $405\ \text{nm}$ (□) and $458\ \text{nm}$ (○) based on the total bilirubin concentration was measured as a function of pH varied by addition of $4\ \text{M}$ -NaOH. The solutions contained $30\ \mu\text{M}$ -bilirubin and $60\ \mu\text{M}$ -albumin in $0.1\ \text{M}$ -NaCl. The symbols ■ and ● denote the measured c.d. at $405\ \text{nm}$ and $458\ \text{nm}$ respectively after approx. 15 min incubation at pH 11.9. The pH was re-adjusted by addition of $1\ \text{M}$ -HCl. The temperature was $26\ ^\circ\text{C}$.

C.d. of bilirubin

The variation with pH of c.d. of the bilirubin-albumin complex (Fig. 4) shows that additional conformational transitions occur in the pH range between 11 and 12, since no plateaus are found. According to Blauer & King (1970) and Blauer *et al.* (1972), the c.d. spectra can be explained by a fixation of the bilirubin molecule in an asymmetric configuration that permits dipole-dipole coupling between the two chromophores. On raising the pH from near neutral to 11.1 the asymmetric twisted bilirubin molecule turns to a symmetric configuration with no c.d. On raising the pH beyond 11.1 the molecule is again twisted, but oppositely to the configuration at more neutral pH. A similar rotation was found at pH 5 by Blauer *et al.* (1972). The maximum c.d. at pH 11.4–11.5 probably reflects a maximum in torsion of the bilirubin molecule. It occurs at the pH where the co-operative dissociation of protons takes place in the first alkaline transition (i.e. at the midpoint of the Hill plot in Fig. 3).

Fig. 5. Fluorescence intensity of bilirubin as a function of pH

The fluorescence of $4\ \mu\text{M}$ -bilirubin ligand to $8\ \mu\text{M}$ -albumin ($\circ$) and of $4\ \mu\text{M}$ free bilirubin ($\bullet$) was measured at 520 nm when excited at 450 nm in 0.1 M-NaCl at different pH values, varied by addition of 1 M-NaOH. The temperature was 25 °C.

Fluorescence of bilirubin

The fluorescence of bilirubin at various pH values is shown in Fig. 5. Without albumin present the fluorescence increases slightly when the pH is raised to 12, probably because of dissociation of the lactam groups with pK values around 12.9 (Hansen *et al.*, 1979). With albumin present it is found that, although the intensity varies, having a minimum at pH 11.6, it is far above the fluorescence of the free form. Bilirubin is thus bound to albumin in the pH range between 11 and 12, confirming the interpretation of the spectral shift in Fig. 1 as being caused by conformational changes in the bilirubin-albumin complex, and not by scission of bilirubin from the complex.

Quenching of albumin fluorescence by bilirubin

In proteins containing both tyrosine and tryptophan residues, as in albumin, the quantum yield of tyrosine emission is very low (Chen, 1967b). The fluorescence pictured in Fig. 6 thus occurs solely from the lone tryptophan residue of albumin. Without bilirubin present the quenching of tryptophan fluorescence at pH 12 relative to that at pH 11 is explained by energy transfer to ionized tyrosine residues (Chen, 1967b). Liganding bilirubin to albumin quenches much of the tryptophan

Fig. 6. Quenching effect of bilirubin on the tryptophan fluorescence of albumin at various pH values

The fluorescence spectra of $4\ \mu\text{M}$ -albumin in 0.1 M-NaCl (—) and of $4\ \mu\text{M}$ -albumin with $2\ \mu\text{M}$ -bilirubin liganded (---) were measured at pH 11 and pH 12 when excited at 290 nm. The temperature was 25 °C.

fluorescence at pH 11 because of energy transfer between tryptophan and bilirubin. Using this phenomenon, Chen (1973) and Berde *et al.* (1979) calculated the distance between them at neutral pH to be 2.7–2.8 nm (27–28 Å). At pH 12, however, the addition of bilirubin quenches almost none of the tryptophan fluorescence. The very low efficiency of energy transfer between the donor and acceptor suggests an increased distance between them (Stryer, 1978).

DISCUSSION

Stability of the bilirubin-albumin complex at extreme alkaline pH

A crucial point in the present work is whether irreversible damage occurs to albumin at these high pH values. It is especially relevant to exclude the formation of A-albumin, dimer and the denatured component that arise when albumin is kept above pH 10 for 96 h (Nikkel & Foster, 1971). The formation of A-albumin occurs at low ionic strength, and is practically blocked in 0.1 M-NaCl as used in the present experiments (Stroupe & Foster, 1973). As revealed by gel electrophoresis, no increase in dimer content, no denatured components and no cleavage products were formed during the short time of the experiments. Concerning the stability of albumin in strong alkaline solutions, experiments including acidimetric titration, u.v.-light-absorption and c.d. measurements all indicated that no damage occurred to the albumin molecule within 15–20 min. However, above approx. pH 11.8 the bilirubin molecule was unstable, probably becoming oxidized. When care was taken to limit exposure times at extreme pH values, reproducible results were obtained, and the spectral changes were reversible. Strong evidence thus indicates that the two

alkaline transitions are reversible in experiments lasting a few minutes.

First alkaline transition

As judged from light-absorption experiments, the first alkaline transition at pH 11.3–11.8 involves a co-operative dissociation of at least six protons (Hill coefficient 6.1–6.6; Fig. 3). This represents a high degree of co-operativity occurring in the bilirubin-albumin complex. The numerical value is somewhat higher than 5 as reported by Brodersen (1979). The origin of the protons is probably tyrosine residues, since Decker & Foster (1967) and Steinhardt & Stocker (1973) found that tyrosine residues in albumin ionize in this pH interval in a co-operative way. Steinhardt & Stocker (1973) suggested that six tyrosine residues deprotonate co-operatively, with pH 11.33 as midpoint. This is very close to our Hill coefficient, and indicates that the conformational change occurs in the bilirubin-albumin complex as well as in the albumin molecule itself. The midpoint of our titration on the bilirubin-albumin complex, pH 11.45, is a little higher than pH 11.33. Bilirubin in low concentrations probably stabilizes the albumin molecule by inhibiting the deprotonation, as also observed with low concentrations of hydrocarbons and detergents (Steinhardt & Stocker, 1973).

Second alkaline transition

This takes place when the pH is raised from 11.8 to 12.0. It is characterized by a slight blue-shift in light-absorption of bound bilirubin, and can be explained by a partial exposure to the solvent of the otherwise buried bilirubin chromophores.

Albumin structure during the alkaline transitions

As proposed by Brown (1978), the albumin molecule consists of six half-domains. On the basis of this model Brodersen (1979) and Jacobsen & Brodersen (1983) have suggested an arrangement whereby the second and third half-domains from the *N*-terminal each bind one bilirubin chromophore, thereby forming one bilirubin-binding site. The change in c.d. induced by change of pH is then explained by a rotation of the two half-domains relative to each other. Above pH 11.1 the two half-domains are twisted oppositely to the conformation at more neutral pH.

The fluorescence experiments, together with the observations of light-absorption, are best explained by reversible unfolding of the albumin molecule in the second alkaline transition, whereby the distance between tryptophan-214 and bilirubin increases, and the liganded bilirubin molecule becomes partly exposed to the solvent.

Conclusion

The results are consistent with a proposed model of albumin consisting of six half-domains of which two combine in forming one bilirubin-binding site (Brodersen,

1979; Jacobsen & Brodersen, 1983). Above pH 11.1 the two half-domains bind the bilirubin molecule, twisted oppositely to the configuration at more neutral pH.

On the basis of light-absorption two alkaline transitions of albumin take place. The first alkaline transition occurs at pH 11.3–11.8, involving a co-operative dissociation of at least six protons. The second alkaline transition occurs at pH 11.8–12.0 and is characterized by a slight blue-shift of the bilirubin spectrum. It probably implies a reversible unfolding of the albumin molecule, partly exposing the liganded bilirubin to the solvent.

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