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Lysine Residue 240 of Human Serum Albumin is Involved in High-Affinity Binding of Bilirubin

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Bilirubin can be coupled covalently to albumin by using water-soluble carbodi-imide as coupling reagent. The optimal specificity in the attachment of bilirubin to the high-affinity site on the albumin molecule was obtained by treating an albumin–bilirubin complex with carbodi-imide in low concentrations and for a short period. The product was reduced, carboxymethylated and digested with trypsin. By fractionation on Sephadex G-50 (superfine grade) a peptide fraction containing most of the bilirubin label was isolated. Further purification by paper chromatography gave one peptide, consisting of residues 240–258. The peptide contained a single lysine residue, 240, and had an intact disulphide bridge. The results indicate that bilirubin is bound to lysine residue 240 at its high-affinity site on human serum albumin.

The chemical nature of the bilirubin-binding site on albumin has previously been studied by measuring the affinity of bilirubin for albumin derivatives obtained by chemical modification of amino acid residues on the albumin molecule. Modification of histidine, arginine and tyrosine residues decreased the binding affinity, whereas modification of a limited number of amino and carboxy groups, the single cysteine and the single tryptophan residues did not change the affinity for bilirubin (Jacobsen, 1972). Chemical modification demonstrated the presence of at least one lysine residue in or near the binding site (Jacobsen, 1975). It has been shown that bilirubin can be coupled covalently to albumin by using water-soluble carbodi-imide as coupling reagent. The linkage is formed between one of the carboxy groups of bilirubin and a reactive lysine residue on albumin (Jacobsen, 1976).

The present work describes an improved carbodi-imide coupling method that has been developed with the intention of achieving a high specificity in the covalent attachment of bilirubin to the high-affinity site of albumin. A tryptic fragment containing bilirubin has been isolated and its location in the final sequence of human serum albumin determined.

Materials and Methods

Materials

Human serum albumin was obtained from AB Kabi, Stockholm, Sweden. The preparation contained 98% serum albumin and 0.5 mol of fatty acids per mol of albumin. Trypsin (EC 3.4.21.4; 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one-treated) was from Worthington Biochemical Corp., Freehold, NJ, U.S.A. [1,2,3,4,5,6,7,8-^{14}C]Bilirubin (sp. radioactivity 20.4 mCi/mmol) was obtained from Amersham Buchler, Braunschweig, Germany. All other reagents were of highest purity commercially obtainable and were used without further purification. Water was demineralized and deaerated before use.

Albumin–bilirubin coupling

The sequential reactions are summarized in Scheme 1. As far as possible all manipulations were performed in the dark and at 20°C unless otherwise stated.

Step (1). An albumin/bilirubin solution with the molar ratio 1:0.7 was prepared in the following way: 150 mg of albumin was dissolved in 1.87 ml of water and pH was adjusted to 7.4 with 1 M-NaOH. Then 1.13 ml of 1.4 mM-[^{14}C]bilirubin, containing 5 μCi, adjusted to pH 8.4 was added. The solution was diluted with water to a final volume of 60 ml, corresponding to a 0.25% (w/v) protein solution. The pH was lowered to 4.75 by adding 1 M-HCl and 150 mg of 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide hydrochloride was added with stirring over a period of 5 min. The pH was maintained at 4.75–5.0 by adding 1 M-NaOH or 1 M-HCl. The reaction was continued for a further 20 min. The quantities used correspond to a molar ratio for reagent/albumin of 344.

Step (2). A portion (60 mg) of active charcoal was suspended in a minimum of water and added to the above solution. Then 15 ml of 0.1 M-sodium phosphate buffer, pH 7.4, containing 0.5 M-sodium salicylate was added. This solution was prepared im-

(1) A·B (1:0.7 complex) $\xrightarrow[\text{pH 4.75; 20°C; 25 min}]{\text{Carbodi-imide treatment}}$

(2) A–B+B, chemically modified $\xrightarrow[\text{pH 7.4; 20°C; 30 min}]{\text{Salicylate/charcoal treatment}}$

(3) A–B $\xrightarrow{\text{Sephadex G-25}}$

(4) Freeze-drying

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(5) A–B $\xrightarrow[\text{6 M-guanidine hydrochloride; pH 8.1; 20°C; 3.5 h}]{\text{Reduction with dithiothreitol}}$

(6) A–B reduced $\xrightarrow[\text{pH 8.1; 20°C; 15 min}]{\textit{S}\text{-carboxymethylation with sodium iodoacetate}}$

(7) A–B reduced; carboxymethylated $\xrightarrow{\text{Sephadex G-25}}$

(8) Freeze-drying

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(9) A–B reduced; carboxymethylated $\xrightarrow[\text{pH 8.3; 37°C; 4 h}]{\text{Trypsin digestion}}$

(10) A–B reduced; carboxymethylated; trypsin-digested $\xrightarrow{\text{Sephadex G-50}}$

(11) Isolation of fractions with high degree of labelling

(12) Further purification by paper chromatography

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(13) Amino acid composition of eluted peptides

(14) Location of the fragment in the primary structure of human serum albumin

Scheme 1. *Reaction scheme of the carbodi-imide-catalysed coupling of bilirubin to the high-affinity site on human serum albumin and the subsequent isolation of the bilirubin-containing fragment*

Abbreviations: A, human serum albumin; B, bilirubin; A–B, bilirubin covalently attached to albumin. The horizontal lines separate the different stages in the process i.e. (1)–(4), albumin–bilirubin coupling; (5)–(8), reduction and carboxymethylation; (9)–(12), trypsin digestion and separation of fragments; (13), amino acid analyses.

mediately before use. After stirring for 15 min, another portion (90 mg) of active charcoal that had been rinsed in water was added and stirring was continued for 15 min. The charcoal was removed by filtration through Whatman GF/A filters.

Steps (3) *and* (4). The solution was passed through a Sephadex G-50 column (5 cm × 50 cm) with water as eluent to remove traces of charcoal, salicylate and residual uncoupled bilirubin. The collected protein was freeze-dried and stored at −15°C.

Reduction and carboxymethylation

Step (5). Reduction of carbodi-imide-treated albumin–bilirubin was accomplished by a procedure described by Waxdal *et al.* (1968). For this 60mg of protein dissolved in 3ml of 0.5M-Tris/HCl buffer, pH 8.1, containing 2mM-EDTA and 6M-guanidine hydrochloride, was transferred into a vial. The vial was flushed with N_2, capped and kept at 50°C for 30min. Dithiothreitol (120mg, corresponding to 50mol/mol of disulphide in the protein) was added. Again the vial was flushed with N_2, capped and shaken for 3.5h at 50°C.

Step (6). Carboxymethylation of the reduced protein was carried out at 20°C by adding sodium iodoacetate in an amount corresponding to 100mol/mol of disulphide in the protein. The sodium iodoacetate was prepared by neutralizing 287mg of iodoacetic acid with 1.54ml of 1M-NaOH. In addition 2ml of the Tris/EDTA/guanidine hydrochloride solution, pH8.1 (see above), was added and the mixture was left for 15min.

Steps (7) *and* (8). The solution was eluted from a Sephadex G-25 column (2.5cm×40cm) with 0.15M-NH_4HCO_3, pH8.3. The protein fraction was collected and freeze-dried.

Trypsin digestion and separation of fragments

Steps (9) *and* (10). Protein (60mg) was dissolved in 3ml of 0.15M-NH_4HCO_3, pH8.3, and to this was added trypsin in the protein/trypsin ratio of 50:1 (w/w). After 4h at 37°C, the solution was placed on a Sephadex G-50 (superfine grade) column (2.5cm×90 cm) and eluted by upward flow with 0.15M-NH_4HCO_3, pH8.3, at 40ml/h: fractions (5ml) of eluate were collected and the A_{220}, A_{230} and A_{278} were measured. The radioactivity was determined on a sample (0.6ml) mixed with 6ml of Instagel (Packard Instrument Co., Downers Grove, IL, U.S.A.) in a Packard Tri- Carb liquid-scintillation spectrometer.

Step (11). By comparing the distribution of radioactivity in the separate fractions with the A_{220}, A_{230} and A_{278}, peptides with a high degree of labelling were identified. The appropriate fractions were pooled and freeze-dried.

Step (12). Descending chromatography was performed on Whatman 3MM paper. As solvent butan-1-ol/pyridine/acetic acid/water (15:10:3:12, by vol.) was used in all runs. A sample (2–10mg) of peptide dissolved in 30–100μl of water was applied in a band of approx. 5cm in length. The chromatograms were developed for 17–25h. After drying, a guide strip was stained with ninhydrin/cadmium reagent to localize peptide bands (Heathcote & Haworth, 1969). Another guide strip, 1cm in width, was divided in pieces of length 1cm, which were cut and placed in counting vials. Water (1.8ml) was added to each, and after shaking for 2h at room temperature, 6ml of Insta-gel was added before radioactivity counting.

The pooled fractions from Sephadex G-50 (superfine grade) as well as eluted peptide bands from chromatograms were analysed for the contents of peptides by dansylation and subsequent paper chromatography (Seiler, 1970). Peptide bands were eluted with water and freeze-dried. The dansylation was performed with a suitable amount of peptide material by treating it with 10μl of 0.1M-$NaHCO_3$, pH9.8, and 10μl of 0.5% dansyl chloride in acetone for 30min at 37°C with stirring. Then 10μl of 10% (v/v) formic acid was added before chromatographing the mixture on Whatman 3MM paper for 18–20h with the solvent mentioned above.

Amino acid analyses

Step (13). The eluted and freeze-dried peptides from paper chromatograms (0.5–1mg) were hydrolysed for 20–24h at 110°C in 1ml of 6M-HCl (twice distilled). The ampoules were flushed with O_2-free N_2 before sealing. HCl was removed by flushing N_2 into the ampoules while heating to 60–70°C. Amino acid analyses were performed with a Beckman Autolab instrument with full deflection at a solution amino acid concentration of 1nM. The analyses were kindly performed by Dr. T. Ellebæk Petersen, Institute of Molecular Biology, University of Aarhus.

Results

Coupling procedure

A method by which bilirubin can be coupled covalently to albumin has been described previously (Jacobsen, 1976). In the present work this method has been improved to obtain the optimal specificity in the coupling. In the albumin–bilirubin complex, less than 1mol of bilirubin/mol of albumin was selected to ensure that all bilirubin was present at the high-affinity site (Jacobsen, 1969). The treatment with carbodi-imide (Scheme 1, step 1) was carried out under mild conditions, at a reagent/albumin molar ratio of 344. The specificity was enhanced by keeping a low protein concentration (0.25%, w/v) in the reaction mixture and a short reaction period. Determination of the free amino groups (Jacobsen, 1975) of the albumin–bilirubin compound revealed that the number was only diminished by 5%, corresponding to three or four amino groups modified.

In the procedure, salicylate displaces unbound bilirubin, which can then be absorbed by the charcoal (Scheme 1, step 2). The experiments showed that approx. 20% of the added [^{14}C]bilirubin was linked covalently under the conditions selected.

Isolation of bilirubin-containing fragments

The first step in the isolation of bilirubin-con-

taining fragments was reduction with dithiothreitol in 6M-guanidine hydrochloride followed by carboxymethylation of the thiol groups formed (Scheme 1, step 6). Later, amino acid analyses showed that not all 17 disulphide bridges in albumin were reduced under these circumstances. Thus the isolated bilirubin–peptide contained an unreduced disulphide bridge.

The cleavage by trypsin of the albumin–bilirubin compound was carried out with an enzyme concentration of 2% (w/w). The specific activity of the albumin–bilirubin compound was so low that isolation by high-voltage electrophoresis after localization of bilirubin-containing peptides by radioautography was not possible. For this reason purification required first high-resolution gel filtration followed by preparative paper chromatography.

Fig. 1(*a*) shows the elution profile of the trypsin-

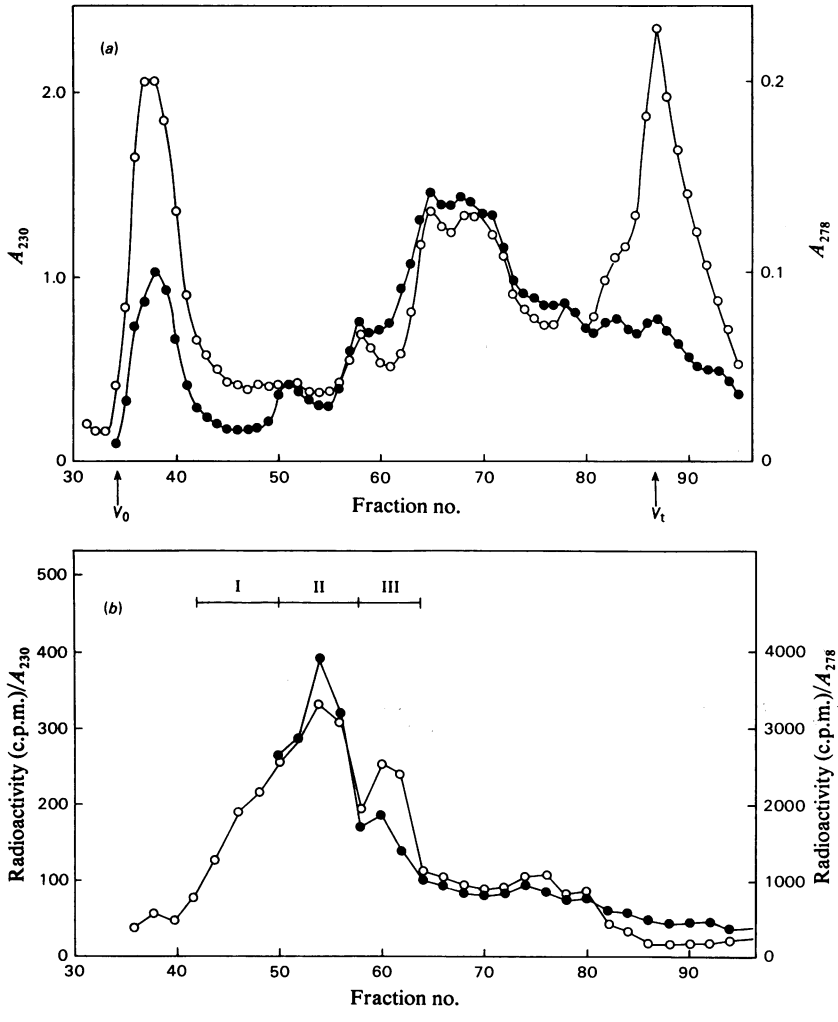

Fig. 1. *Elution profile of trypsin-digested, reduced and carboxymethylated carbodi-imide-treated albumin–[^{14}C]bilirubin (1:0.7, complex) from a Sephadex G-50 (superfine grade) column (2.5cm×90cm)*

Elution was performed with 0.15M-NH_4HCO_3, pH8.3. The effluent was collected in fractions (5ml). V_0 is the void volume and V_t is the total volume of the column. (*a*) Absorbance at 230nm (●) and 278nm (○). (*b*) Relative distribution of label, [^{14}C]bilirubin, in the fractions. The radioactivity was measured on a sample (600μl) dissolved in 6ml of Insta-gel. Bars (I)–(III) denote the fractions that were pooled. Absorbance was measured at 230nm (●) and 278nm (○).

digested protein on a Sephadex G-50 (superfine grade) column. The peptide content in the fractions was determined by measuring A_{220}, A_{230} and A_{278}. The A_{220} and A_{230} showed almost identical patterns, whereas the A_{278}, which is due to the presence of tryptophan and tyrosine residues, was relatively high at the void volume and at the total volume.

Fig. 1(*b*) shows the relative content of label in the individual fractions. The content was determined by calculating the ratio of radioactivity to A_{230} or A_{278} for each fraction. It appears from Fig. 1(*b*) that one peptide fraction exceeds the others in extent of labelling; this fraction was eluted at fraction 54. Fractions 50–58 were accordingly pooled and termed fraction (II). Correspondingly fractions 42–49 and fractions 59–64 constitute fractions (I) and (III), respectively.

Samples (2–10mg of each) of the freeze-dried fractions (I), (II) and (III) were subjected to paper chromatography. Fig. 2 shows the relative distributions of radioactivity, obtained by counting on 1cm × 1cm guide strips. Only fraction (II) contained peptides with a high content of [^{14}C]bilirubin. Most of the bilirubin was attached to a peptide located approx. 5cm from the origin. Peptide bands 4–8 and 14–18 of fraction (II) (denoted by bars in Fig. 2) were eluted and freeze-dried. A sample of each was dansylated and checked for well-defined peptides. Results from three separate experiments showed that the dansyl-peptides from bands 14–18 were not well-defined. Therefore, no further analyses were performed on the eluates from bands 14–18. Bands 4–8, however, showed one strong fluorescent spot with *R* 0.40 relative to 5-dimethylaminonaphthalene-1-sulphonic acid. The content of a well-defined peptide in fraction (II) was confirmed by dansylation of samples of fractions (I), (II) and (III). Only fraction (II) showed well-defined dansyl-peptide spots on subsequent paper chromatography. The *R* values were in agreement with that found for bands 4–8. A strong fluorescent spot was found at *R* 0.40 and a very weak one at *R* 0.20.

The peptide located at bands 4–8 was eluted and subjected to hydrolysis in 6M-HCl for 24h at 11°C. Subsequent amino acid analyses showed that the amino acid content of the fragment agreed with the amino acid composition between positions 240 and 258 in the final sequence of human serum albumin given by Meloun *et al.* (1975) [positions 239–257, in the sequence published by Behrens *et al.* (1975)]. The results are summarized in Table 1. The minor deviations from integer values may be due to traces of other peptides present in bands 4–8. The molecular weight of the peptide was calculated to be 2115. A value within the range 2000–3000 would be expected from the elution volumes of the peptide on Sephadex G-50. The peptide contained one cystine residue, although the original protein was reduced and carboxymethylated. This could be explained by assum-

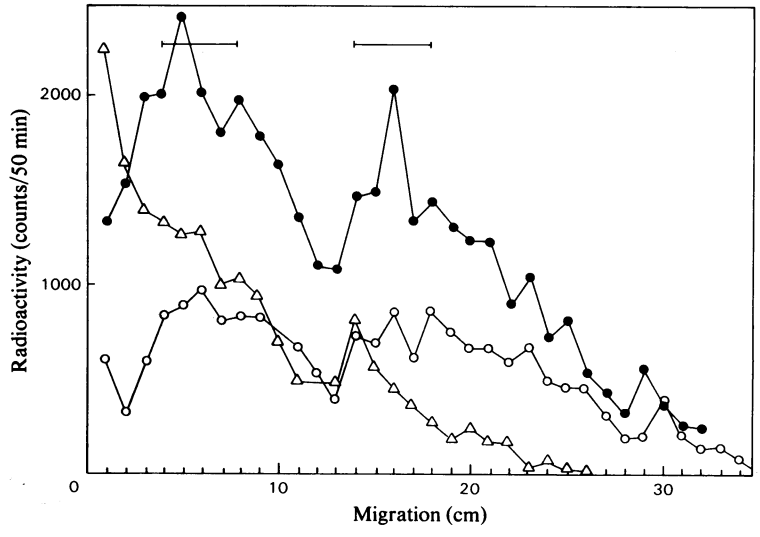

Fig. 2. *Descending paper chromatography of the pooled fractions (I)–(III) in Fig. 1(b)*
The peptide material was applied in a band of 5cm length. A guide strip, 1cm wide was divided in pieces of 1cm length and suspended in a mixture of 1.8ml of water and 6ml of Insta-gel. The bars denote the labelled peptides that were eluted and subjected to further analysis. Symbols: △, fraction (I); ●, fraction (II); ○, fraction (III).

Table 1. *Amino acid composition of the bilirubin-containing fragment*

The fragment was obtained by trypsin digestion of reduced and carboxymethylated human serum albumin at which bilirubin was linked covalently. Values for the fragment were calculated relative to alanine, assuming 2.0 alanine residues/molecule of fragment (Meloun *et al.*, 1975). Values are mean values of double determinations. For comparison, the appropriate composition found by Meloun *et al.* (1975) is shown for the fragment. No corrections for losses were made. Total number of residues 19; calculated mol.wt. 2115.

Amino acid	Content (residues/molecule of fragment)		Predicted composition (residues 240–258) Data of Meloun *et al.* (1975)
	Present work		
Alanine	2.0	2.0	2
Arginine	0.7	0.7	1
Aspartic acid	2.8	2.8	3
Carboxymethylcysteine	0.4	0.3	3
Half-cystine	2.0	2.0	
Glutamic acid	2.9	2.9	2
Glycine	1.1	1.1	1
Histidine	1.3	1.3	2
Isoleucine	0.0	0.0	0
Leucine	2.0	1.9	2
Lysine	0.9	0.9	1
Methionine	0.4	0.1	0
Phenylalanine	0.3	0.3	0
Proline	0.0	0.0	0
Serine	0.5	0.5	0
Threonine	1.0	1.1	1
Tyrosine	0.0	0.0	0
Valine	0.7	0.8	1

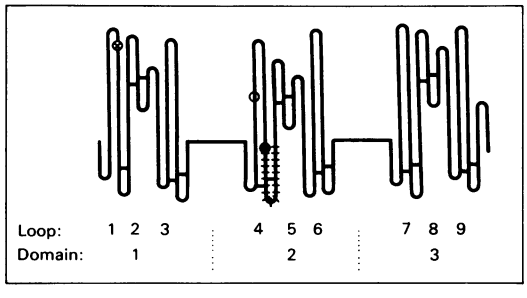

Fig. 3. *Model of human serum albumin from the sequence of Meloun et al.* (1975) *with disulphide bridging according to Behrens et al.* (1975)

Symbols: ●, Lysine-240; ○, tryptophan-214; ⊗, cysteine-34. The hatched peptide from lysine-240 denotes the isolated peptide 240–258.

ing that the disulphide bridge between residues 245 and 253 had resisted reduction by dithiothreitol (see Fig. 3). The fragment isolated only contained one lysine, namely residue 240.

The amino acid composition of the isolated peptide was checked against the complete sequence of albumin, and no alternative locations were found.

Discussion

Chemical basis of the method

The present work is based on the following previously published observations. First, one molecule of bilirubin is bound with very high affinity to a specific site on the albumin molecule (Jacobsen, 1969; Brodersen & Funding, 1977). Secondly, at least one reactive amino group of lysine is located in or near the high-affinity bilirubin site on human serum albumin (Jacobsen, 1975). Thirdly, bilirubin can be coupled covalently to an amino group on albumin by using a water-soluble carbodi-imide as coupling reagent (Jacobsen, 1976).

Bilirubin was bound to albumin at pH 8. Then lowering the pH to 4.7–5.0 meant that one of the carboxy groups of bilirubin could be coupled to an amino group of lysine after activation with carbodi-imide. A high specificity in the attachment (i.e. maximal coupling of bilirubin to a single lysine residue in or near the binding site) was achieved by using: (1) less than one mol of bilirubin/mol of albumin; (2) low protein concentration (0.25%, w/v); (3) a small excess of carbodi-imide; (4) a short reaction time.

Although the reaction was carried out at pH 5, it can be assumed that the albumin structure remains

compact (unexpanded) (Wallevik, 1973), leaving the bilirubin site intact. It is most likely that bilirubin is coupled to lysine-240, as only this lysine residue was present in the peptide isolated.

Bilirubin-binding site on albumin

Two slightly different sequences of human serum albumin have been published, by Meloun *et al.* (1975) and by Behrens *et al.* (1975). In the albumin model of Brown (1975) and Behrens *et al.* (1975), the disulphide bridging bends the peptide chain into nine double loops. Experimental findings also support the view that association of the nine loops forms globular parts or domains (Anderson & Weber, 1969; Peters, 1975). Loops 1–3 constitute domain 1, loops 4–6 domain 2 and loops 7–9 domain 3. An albumin model based on loops and domains is shown in Fig. 3.

The lysine residue 240 that linked with bilirubin is located in the middle of loop 4 (Fig. 3). Coupling to that lysine residue can be explained if bilirubin is bound primarily in a cleft formed in a region of either loops 3–4 or loops 4–6. One of the bilirubin carboxylate groups may, after activation with carbodi-imide, react with an amino group in a favourable position, in the present case an amino group located in the middle of loop 4. It has been demonstrated that salt linkages as well as hydrogen bonds make a major contribution to the bilirubin binding (Jacobsen, 1977). It is therefore likely that lysine-240 is the residue that is bound to one of the carboxylate groups of bilirubin with a salt linkage. The other carboxylate group of bilirubin is probably involved in another salt linkage to a lysine or arginine residue (Jacobsen, 1972). The high net-positive charge at pH 7 of loop 4 in contrast with other loops may be important in these reactions.

Reed *et al.* (1975) have demonstrated that the high-affinity bilirubin site on bovine serum albumin involves the residues 186–238. This result was found by measuring the affinity of bilirubin for fragments of bovine serum albumin obtained by limited proteolysis. They suggested that bilirubin is bound in the cleft formed by domains 1 and 2, as the fragment composed of residues 186–238 is located in that region of the molecule (Reed *et al.*, 1975; Peters, 1975).

Geisow & Beaven (1977) found that bilirubin binds to two large fragments of human serum albumin. They consist of residues 1–386 and residues 49–307. The complexes between the fragments and bilirubin showed similar circular-dichroism spectra to that of the complex between bilirubin and whole albumin. Gitzelmann-Cumarasamy *et al.*, (1976) have identified two human serum albumin peptides, consisting of residues 124–297 and 446–547, which become covalently labelled with a reactive bilirubin derivative.

These observations together with the present findings suggest that the primary site for bilirubin is located in the region of loops 3–4 of the albumin molecule.

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