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FLUORESCENCE AND STOPPED-FLOW STUDIES ON THE N ⇌ F TRANSITION OF SERUMALBUMIN

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In order to characterize the isomerization of serumalbumin in the acidic pH-range equilibrium and kinetic measurements of the intrinsic fluorescence of bovine serumalbumin and human serumalbumin were performed. Additional experiments with modified bovine serumalbumin made use of substituted 1.9 benzoxanthene dyes as SH-specific extrinsic fluorophores. The intrinsic fluorescence (λ_{exc} = 275 nm) shows a pH-dependent shift of the maximum of fluorescence emission which correlates with the N ⇌ F isomerization. This and the acid expansion at pH < 3.5 is indicated by the pH-dependence of the fluorescence intensity at 350 nm. While tyrosine fluorescence is increased in all steps of the transition, tryptophane fluorescence is decreased in a different way for BSA (2 trp/molecule) and HSA (1 trp/molecule), the latter showing the N ⇌ F transition only. Combining the tryptophan fluorescence data with the results from the SH-specific modification of BSA the conclusion may be drawn that the tryptophan residues in BSA and the SH-group belong to different domains of the molecule. Stopped-flow experiments prove the N ⇌ F' and the F' ⇌ F transitions to be separable along the time axis, the relaxation times being in the range between 40–50 and 300–600 msec respectively. For the "expansion" the kinetic constants critically depend on the initial pH conditions of the solutions. The backward reaction F → N seems to be a multistep isomerization process which is characterized by relaxation times >1 sec.

1. Introduction*

The native structure of proteins represents the kinetically accessible minimum of the free energy of the intramolecular interactions [1]. As shown by Anfinsen and his coworkers [2–4] the amino-acid sequence contains the total information required for the formation of the three-dimensional structure in a given solvent. However, the mechanism by which the biologically active structure is formed after release of the polypeptide chain from the ribosome is still not fully understood. Some evidence points to the folding of independent domains during chain elongation while refolding experiments seem to prove the two-state model to be sufficient to describe the process of structure formation (for reviews see [1,5]).

Serumalbumin is a suitable model for studying the folding of proteins since it contains a relatively small number of chromophores or fluorophores which show significant alterations of their spectral properties following changes in their environment in the course of the characteristic N ⇌ F isomerization in the acid pH range [6–8].

On the other hand, certain functional groups, e.g. the sulfhydryl group, may be chemically modified by specific fluorescence labels providing additional conformational probes. This way independent analysis of different parts of the molecule is rendered possible.

Serumalbumin consists of a single polypeptide chain (M = 68000 ± 3000) the three-dimensional structure of which is stabilized by 17–19 disulfide bridges. While human serumalbumin (HSA) contains a single tryptophanyl residue, bovine serumalbumin (BSA) contains two. This allows spectral changes to be attributed to different domains of the molecule [8–10]. As shown by earlier hydrodynamic and spectroscopic studies [8,11] the N ⇌ F isomerization in the acid pH range is fully reversible. Including the "expansion" of the molecule to a state S at strongly acidic pH (<3.5) we are dealing with an unfolding–refolding process which may be analyzed using fluores-

* Abbreviations: BSA: bovine serumalbumin, HSA: human serumalbumin, trp: tryptophan, tyr: tyrosine.

cence emission as a conformational probe.

Preliminary kinetic data using solvent perturbation of anomalous tyr residues as a conformational probe indicated the reaction rate to be strongly pH-dependent [12,13].

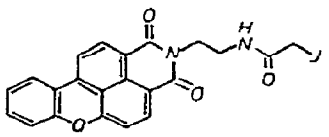
The present results of equilibrium and rapid kinetic experiments are in accordance with earlier fluorescence data [6]. Comparing the data for different serum-albumins the observed spectral changes may be attributed to certain steps of a sequence of isomerization reactions in both the N ⇌ F transition and the acid expansion of the molecule. This result of equilibrium measurements is confirmed by a series of first-order rate processes with relaxation times $40 < \tau < 600$ msec obtained from kinetic experiments using the stopped-flow technique.

2. Materials and methods

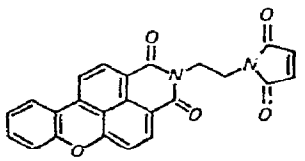
Crystallized BSA and HSA were obtained from Behring-Werke, Marburg/Lahn [Op. Nrs. 62 (BSA), 1887 (HSA)], bovine mercaptalbumin from ICN Nutritional, Cleveland, Ohio [Op. Nr. 8927]. In a number of experiments preparations defatted by charcoal treatment [7] were used. Protein concentration was determined spectrophotometrically using matched quartz cuvettes of 1–10 mm path length (cf. table 1).

SH-determinations made use of the methods of Janatová et al. [14] and Habeeb [15]. To further characterize the proteins, the amount of dimer was estimated by ultracentrifugation and gel chromatography (Sephadex G 100); in addition, fluorescence emission spectra were used for analyzing the purity of the preparations.

Fluorescence labelling of SH groups made use of two 1.9 benzoxanthene dyes (F1 and F2) kindly provided to us by Dr. H. Fasold, Frankfurt/Main:



F1



F2

The albumin solutions in 0.02M NaCl, pH 6.5 were stirred at 4°C with a 4-fold molar excess of F1 or F2 in dimethyl–formamide [final concentration <9% (v/v)] in the absence of air for 12 hours. After separating excess of dye by gel chromatography on Sephadex G 25 and millipore filtration (0.45 μ) the solutions of the modified proteins were adjusted to the desired pH and Cl^- concentration. From the absence of free SH in BSA·F1 after chemical modification and the rough equivalence of the amount of dye bound with the free SH before modification we conclude that only cysteine was modified in this derivative (cf. table 1). In the other products unspecific labelling occurred under all experimental conditions used [16]. Therefore only BSA·F1 will be discussed.

Table 1
Data characterizing different serumalbumin preparations[a]

	$A^{1\%}_{279}$ (1 cm)	$s_{20,w}(S)$	%monomers	Mol SH per mol of albumin	Mol dye per mol of albumin		$\lambda_{em,max}$ (nm)
					F1	F2	
BSA	6.67	4.25(4.28)	84(85)[b]	0.49(0.2)	0.39	1.28	339(339)
HSA	5.30	4.42(4.59)	98(98)	0.06(0.04)	0.91	1.77	333(333)

a) Figures in brackets ( ) refer to defatted samples.
b) Identical results were obtained by gel chromatography (Sephadex G 100, 25 mM phosphate buffer pH 7, 0.2 mM DTT, 1 mM EDTA).

Buffers and all other common reagents were of A-grade purity. DTNB (Ellman's reagent) was purchased from Boehringer, Mannheim; L-glutathione(SH) (>98%) from Serva, Heidelberg. Quartz double-distilled water was used as solvent throughout.

UV absorption was measured in a Zeiss PMQ II spectrophotometer. Fluorescence measurements were carried out using a Hitachi Perkin–Elmer MPF 2A spectrofluorimeter with thermostated cuvette holder and square non-fluorescent 1.00 cm cells. (Hitachi Perkin–Elmer and Hellma, Müllheim, B.) No filters and identical slit widths (5.8 and 10 mm) were applied in the excitation and emission compartment. The recorded spectra were corrected for light scattering and Raman emission but not for wavelength dependence of the detector system. Fluorescence was measured at $\lambda_{exc} = 275$ nm.

For kinetic fluorescence experiments, a Durrum–Gibson stopped-flow spectrophotometer with a Durrum 16 400 fluorescence attachment (pathlength of reaction cell 20 mm) and Xenon lamp (75 W) was applied. Voltage of the photomultiplier was 600 V. Kinetic traces were recorded using a Hewlett–Packard Oscilloscope 181/AR with a Dumont–Land Polaroid Camera. The excitation wavelength was $\lambda_{exc} = 280$ nm, the slit width, 1.0 mm $\hat{=}$ 4.1 nm. The dead time of the stopped flow device was determined to be 1.6 msec. In the stopped-flow experiments the contribution of wavelengths $\lambda_{em} < 355$ nm to the integral fluorescence emission was cut off by a UV filter (Corning 0-52). Since the trp emission at longer wavelength was sensitive to pH changes this experimental set-up seems appropriate to follow the pH-induced reactions. The observed signal (F_{int}) reflects the integrated emission at $\lambda_{em} > 355$ nm which can be compared with the integrated emission over the same wavelength range obtained from static fluorescence spectra.

Protein concentrations in the storage syringe were 3×10^{-6} M in 0.02M NaCl; similarly, the Cl^- concentration of the solvents causing the pH change was kept constant (0.02N) in all experiments. pH was adjusted by small amounts of 0.02N HCl and NaOH, respectively. Temperature was kept constant at 20°C. Numerical data represent average values from at least four experiments.

Sedimentation-velocity runs were performed in an analytical ultracentrifuge (Beckman, Model E) with Schlieren optics and a high-sensitivity photoelectric scanning system using 12 mm double sector cells. To compare normal and charcoal-treated, defatted samples, runs with normal and wedge cells were performed.

3. Results

3.1. Characterization of the native state

Data characterizing the native state of the different albumin preparations in the untreated and defatted state, and after fluorescence labelling with F1 and F2 are given in table 1. As indicated by the last column the fluorescence emission spectra of all samples remain unchanged upon defatting by charcoal treatment suggesting negligible contents of fatty acids in the starting material [7]. The fluorescence spectra ($\lambda_{exc} = 275$ nm) were found to be similar to those reported by Chen [8] indicating a maximum (333 nm) and shoulder (304 nm) of comparable intensity for HSA (pH 6) and a maximum (339 nm) and shoulder (300 nm) of different intensity for BSA, respectively. The fluorescent labels F1 and F2 show an intense absorbance at $\lambda > 400$ nm and a small contribution in the 300–350 nm region capable of transferring energy from donor trp molecules. Their extrinsic fluorescence at $\lambda > 450$ nm was merely used for measuring pH effects. Therefore a detailed description of the spectra is not essential in this context (for details see [16]).

3.2. Fluorescence titration

The pH profile of the *intrinsic* fluorescence of tyr and trp at $\lambda_{em} = 310$ and 350 nm (cf. [8]) is given in fig. 1. The observed changes of the relative fluorescence intensity correspond to a blue shift of the maximum emission $\lambda_{max} = 339 \rightarrow 325$ nm for BSA and $\lambda_{max} = 333 \rightarrow 324$ nm for HSA (fig. 2). The observed pH dependent changes of the relative fluorescence intensities and the maximum emission are in accordance with a blue shift and a decrease of the quantum yield for trp and an increase in the quantum yield for tyr [8]. For BSA the decrease of the trp fluorescence intensity at 350 nm appears not to follow a single ionisation step but exhibits a shoulder at pH < 3.5. Estimates on the basis of relative quantum yields of trp and tyr at 350 nm (cf. [17]) indicate that this second

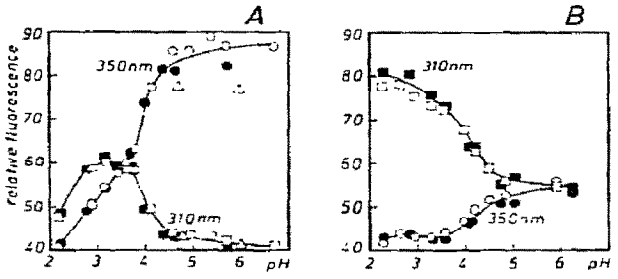


Fig. 1. pH dependence of intrinsic fluorescence intensities of (A) bovine and (B) human serumalbumin. Protein concentration: 3 μM, 0.02N Cl^-, 20°C. Filled symbols: commercial samples; open symbols: defatted samples; △: reverse titration after 2 hours of incubation at pH 2.2–2.8. Wavelength of excitation: λ_{exc} = 275 nm.

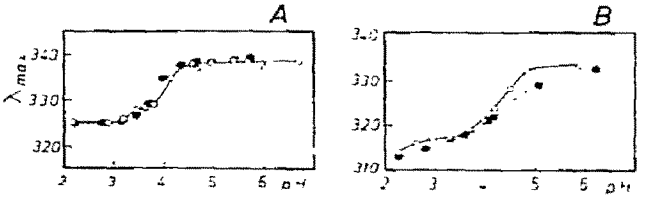


Fig. 2. pH dependence of maximum fluorescence of (A) bovine and (B) human serumalbumin. Conditions and symbols as given in fig. 1.

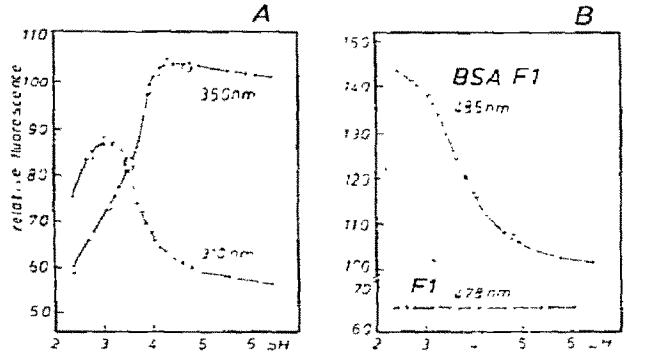


Fig. 3. pH dependence of fluorescence intensity of bovine serumalbumin labelled with F1. A: Intrinsic fluorescence of 1.5 μM BSA·F1 in 0.02N Cl^-, 20°C, λ_{exc} = 275 nm; B: BSA·F1: Extrinsic fluorescence of F1 bound to BSA, 0.02N Cl^-, 20°C, λ_{exc} = 440 nm. F1:1.6 μM F1 in water–dioxane 1:1 (v/v), 20°C, λ_{exc} = 430 nm.

step is not due to residual tyr fluorescence but is associated with the acid expansion of the serumalbumin molecule.

The pH-dependence of the *extrinsic* fluorescence of F1-labelled BSA is given in fig. 3. The fluorescence of the dye itself is independent of the pH. The pH dependence for the label linked to the protein resembles to a certain extent the pH dependence of the intrinsic tyr fluorescence but not that of the trp emission (fig. 3). The apparent coincidence seems to reflect the same structural changes seen by the tyrosines on one hand, and the label on the other. Comparing the properties of BSA and BSA·F1 regarding their maximum of fluorescence emission at pH 6 and the pH dependence of their tyr and trp fluorescence, no significant differences were observed. Blocking of cysteine with F1 as fluorescence label apparently does not interfere with the aromatic residues responsible for the intrinsic fluorescence properties. Contrary to this result the BSA·F2 complex shows drastic changes of the intrinsic fluorescence as expected from the relatively high amount of unspecific labelling which may involve tyr residues. In a similar way the intrinsic fluorescence characteristics of HSA·F1 are altered such that the fluorescence of trp is quenched considerably, λ_{max} showing almost no pH dependence anymore (for details see [16]).

Data in figs. 1–3 represent equilibrium values. The change of fluorescence after addition of HCl or NaOH is rapid. No time-dependent changes were observed on collecting the spectral data. The intensities of fluorescence and the λ_{max} – values as the proteins are titrated from pH 6.5 to pH ≈ 2.2 and back to pH 4.8 or 6.0 are superposable as far as the tyr fluorescence is concerned. In the case of the trp fluorescence irreversible changes of the relative intensity of up to 5% were observed only after long incubation (2 hr) at pH ≈ 2.5. Neglecting this effect, which may be explained by partial photolysis of trp, the N ⇌ F isomerization and the acid expansion may be considered thermodynamically reversible processes [18]. Their cooperative nature which is suggested by the steepness of the transitions is clearly shown by Hill coefficients $n > 1$ as determined from data in fig. 2.

3.3. *Stopped-flow kinetics*

As shown by Chen [6–8] and by the present results,

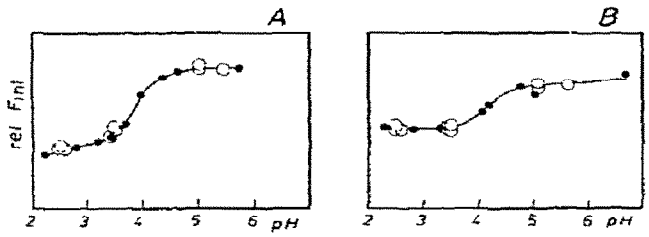


Fig. 4. Comparison of the stopped-flow fluorescence signal (○) with the integrated fluorescence intensities F_{int} from equilibrium measurements (●). For experimental details see sect. 2. Intensities were corrected for absorbance of incident light [19]. Protein concentrations: 1.5 μM (stopped-flow) and 3.0 μM (equilibrium measurements). A: Bovine serumalbumin, B: Human serumalbumin.

the fluorescence of tyr and trp may be used to follow conformational changes in specific domains of HSA and BSA. According to fig. 4 the static fluorescence of BSA shows a characteristic two-step profile in the relative yield of fluorescence (F_{int}, $\lambda_{em} > 355$ nm) which allows the separation of two processes at pH 2.5 → 3.5 and pH 3.5 → 4.5, respectively. For HSA, only the latter process is detectable. The same figure shows the pH profile of the relative yield of fluorescence as obtained from stopped-flow experiments after attainment of equilibrium following the pH jump. The coincidence of both sets of data in the given pH profile indicates that the results of both techniques can be compared.

Using the stopped-flow technique the rates of the isomerization processes underlying the aforementioned spectral changes were studied for both unfolding and refolding (pH 5.0 ⇌ 3.5; pH 5.0 ⇌ 2.5; pH 3.5 ⇌ 2.5). Fig. 5a gives the traces of representative experiments for HSA. Concentration-dependent measurements (cf. HSA, pH 5.0 → 3.4, table 2) show the reaction to obey 1st order kinetics, as expected for an isomerization process. However, plotting each of the relaxation curves in fig. 5 as a 1st order rate process proves one single rate constant to be insufficient to describe the time course of the respective reactions (fig. 5, below). Since the present investigation aims primarily at a correlation between the folding reactions as monitored by static fluorescence measurements, and the respective kinetic processes, the following approach was used to evaluate the relaxation times. In cases where subsequent reactions were resolved by relaxation times $\tau_2 \gtrsim 10\tau_1$ their τ values were determined as if they were independent first-order processes, namely by evaluating the initial and final slopes of the curves in the $\log \Delta F_{int}$ versus time plots. In the other cases the same procedure was applied only to provide an estimate of the magnitude of the τ values. Results for HSA and BSA are summarized in table 2.

In general, unfolding after protonation turns out to be more rapid than refolding after readjustment of the solvent to pH > 5.

Different buffer ions seem to be of minor importance in the time course of the isomerization reaction.

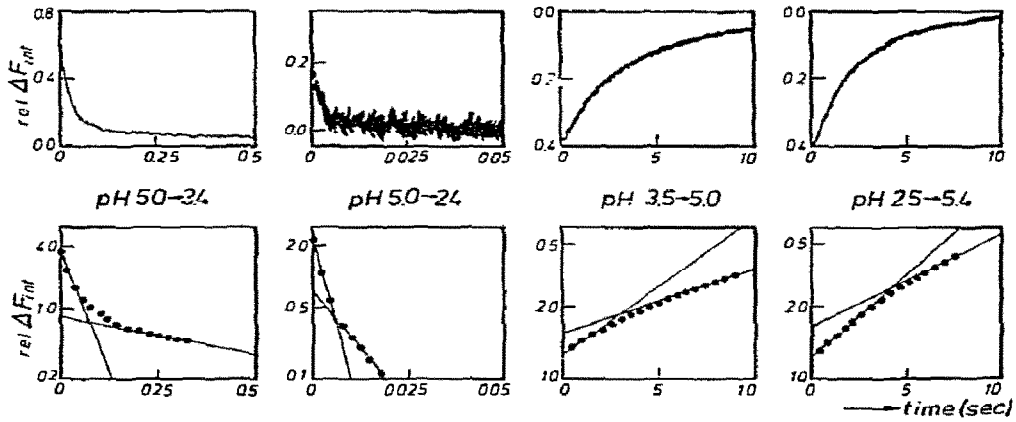


Fig. 5. Representative traces and semi-logarithmic plots of stopped-flow measurements for *human serumalbumin* (HSA). For experimental details see sect. 2.

Table 2
Relaxation times of the pH-dependent transitions of BSA and HSA. (c = 3 μM, 0.02N Cl^-, 20°C, λ_{exc} = 280 nm, $\lambda_{em} \geq$ 350 nm)

pH		BSA		HSA		
Initial	Final	τ_1 (sec)	τ_2 (sec)	τ_1 (sec)	τ_2 (sec)	
5.0	3.4	0.043	0.31	0.055	0.71	(1.5 μM)
				0.052 ± 0.004	0.62 ± 0.05	(3.0 μM)
				0.066	0.65	(6.0 μM)
5.0	2.4	0.005 c)	0.011 a,c)	0.033 c)	0.010 c)	
3.5	2.4	0.11		b)	b)	
2.5	3.5	0.13 c)	0.58 c)	b)	b)	
2.5	5.4	1.9 c)	10	2.7 c)	5 c)	
3.5	5.1	1.4 c)	2.4 c)	3 c)	7 c)	

a) τ_1, τ_2 are followed by a slow third relaxation process (τ_3 = 3.4 sec) of comparably small amplitude (< 20%).
b) No process detectable.
c) Estimate of the magnitude of the apparent relaxation times (see subsect. 3.3).

Results of stopped-flow experiments using phosphate buffer, pH 5.5, acetate buffer, pH 3.7, and 0.01 HCl, pH 2, are in qualitative agreement with the results given in table 2. The same holds for experiments with different protein preparations.

4. Discussion

The acid-induced structural changes of serum-albumin represent a reversible conformational disruption of the compact native molecule to an expanded state characterized by the unmasking of groups initially inaccessible to the solvent (cf. [11–13,20,21]). As suggested by a great variety of experimental techniques, including fluorescence, the whole isomerization reaction may be described as a cooperative process consisting of at least three consecutive steps which finally lead to an increased independence of certain domains of the molecule accompanied by a partial helix–coil transition [22]. The cooperativity is proved by the steepness of the transitions as contrasted by the normal pH range of ionization of independent ionizable groups according to the Henderson–Hasselbalch equation.

Since there is only one trp in HSA and two in BSA the pH-dependent isomerization may be analyzed in terms of changes of the local environment of this intrinsic fluorescent probe. Indirect evidence [23] suggests one of the trp residues in BSA to be exposed to the solvent, while the other one (like the one in HSA) is in an unpolar environment in the inner part of the molecule [24,25].

In the schematic representation of the BSA molecule suggested by partial proteolysis [9,10] 1 trp, 8 out of 18 tyr and the cysteine residue are situated in one domain representing about one third of the molecule; the second trp plus 2 more tyr lie in a second domain while the rest of the tyr residues are distributed over the remaining two domains. From solvent perturbation spectra [26] and pH-dependent absorption at 243 nm [12] the number of tyr residues inaccessible in the native state of HSA is estimated to be 40–60%. These become gradually accessible in the N → F isomerization and the acid expansion.

In the case of the *intrinsic* fluorescence of BSA, fig. 1 shows two steps to be separable in the pH dependence of the fluorescence intensity while HSA shows only a single transition. Comparing the pH profile of HSA and BSA we conclude that one trp residue responsible for the quenching of the fluorescence in the N → F transition must be situated in a similar local environment in both proteins. This confirms the predictions from earlier spectral results mentioned before. Contrary to the fluorescence change of the trp residue common to both HSA and BSA, the fluorescence of the additional trp in BSA is quenched in the course of acid expansion. No unequivocal explanation can be given for both the blue shift and the decrease of the trp fluorescence in the N → F transition (cf. [6,8,24,27]).

In the case of the pH dependence of the *extrinsic* fluorescence of BSA · F1 *no changes* of the positions of the maxima of excitation and emission are observed upon specifically labelling the SH group in BSA. Similarly, the pH dependence of the *intrinsic* fluorescence of BSA · F1 is identical to the one in the native BSA molecule. This leads us to believe that the SH group in BSA cannot be in direct neighbourhood to the trp residue functioning as a conformational probe in the N → F transition.

Regarding the kinetics of the acid-induced isomerization of BSA a variety of methods have been applied. Kessler and Dunn [28] found one very short relaxation time ($\tau = 7.2 \times 10^{-8}$ sec) at pH > 4.3 which might be correlated to the protonation of carboxylic groups. Takahashi and Alberty [29,30] reported similar results on the basis of pressure-jump experiments; in addition, they observed two more relaxation times (40 and 200 msec) in the pH range pH 2.8–3.8. Temperature-jump studies in this pH range led to $\tau \approx$ 20 msec (cf. [29]). Preliminary stopped-flow experiments on the reverse reaction (pH 3.8 → pH > 10) indicate complex kinetics [12]. None of these experiments allowed a correlation of the given relaxation times to specific isomerization steps.

In the present experiments, as in most earlier investigations the isomerization is confirmed by the fact that the overall rate of the refolding reaction is independent of protein concentration. On the other hand, the kinetic traces of the elementary steps of the isomerization do not fit half logarithmic plots for single 1st order processes. This finding clearly contradicts a simple all-or-none mechanism in the given pH-jump stopped-flow experiments.

There might be some ambiguity regarding the kinetic data arising from heterogeneity in SH content and/or the content of fatty acids or higher aggregates of the BSA molecule. Significant effects of these phenomena may be excluded because of negative results of the corresponding control experiments including experiments with commercial mercaptalbumin. We cannot rule out, however, effects of microheterogeneity [31] which might cause relaxation *spectra* instead of well defined relaxation *times*. If we assume the heterogeneous species to differ only slightly such that "broadening" of the relaxation times may be neglected, the following reaction scheme (fig. 6) may be proposed to explain the present kinetic data (cf. [11]).

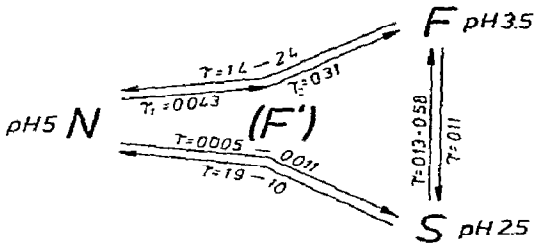


Fig. 6. Reaction scheme for the kinetics of the isomerization of *BSA*. Relaxation times in sec (cf. table 2).

Excepting the acidic transition which remains undetected in the fluorescence emission of HSA (fig. 4), both HSA and BSA show comparable kinetic results. The given rate constants can be correlated to the stepwise separation of different domains of the molecules. In this context, changes of the polarity in the local environment of the fluorophores may be tentatively derived from the spectral shifts accompanying isomerization. Accordingly the local environment of one trp residue in BSA and HSA (the fluorescence of which is quenched in the N → F transition) must be comparable. The trp specific blue shift which can be measured directly by excitation at 295 nm [8] in this case indicates enhanced hydrophobic interactions allowed by the increased flexibility of the polypeptide chain in the F state of the molecule.

Considering the kinetics of the pH-dependent isomerization, the pH jump pH 5 → 3.4 leads to two relaxation times similar to those obtained from pressure-jump experiments [29]. The respective reverse reaction is about 10 times slower, i.e. the microscopic rate constants for N → F and F → F′ differ considerably. However, the effect is within the range of normal differences in the activation energy and may be explained by entropic effects in the refolding process as well.

For the F → N and S → N transitions comparable relaxation times were measured. They may be indicative of a common intermediate. In the acid expansion of BSA (F → S) a slow reaction ($\tau \approx 0.1$ sec) takes place which is not observed in the case of the N → S transition. Apparently the F state is not realized in this reaction; otherwise the slow process should contribute as rate-determining step. From this we exclude a sequential mechanism N → F → S. Instead, we propose the branched mechanism (fig. 6) which includes

the F' state as a common intermediate of the N → F and N → S transitions. A similar intermediate has been proposed earlier by Foster [11]. The shorter relaxation time of the N → S transition as compared to the N → F transition may be explained by the higher electrostatic repulsion of charges in the fully protonated molecule.

The branch mechanism is similar to Tanford's denaturation mechanism describing a biphasic 1st order kinetic plot [32]. Whether the well known restrictions regarding cyclic equilibria [33] hold in the case of the N → F transition and the acid expansion remains to be analyzed. In the interpretation of the kinetic data each of the equilibria corresponds to a separate relaxation process, the initial and final states belonging to different conformers characterized by their different net charge and by specific spectral properties.

Acknowledgements

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