Stopped-flow kinetic analysis of long-chain fatty acid dissociation from bovine serum albumin

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The kinetics of the interaction of long-chain fatty acids (referred to as fatty acids) with albumin is critical to understanding the role of albumin in fatty acid transport. In this study we have determined the kinetics of fatty acid dissociation from BSA and the BSA-related fatty acid probe BSA-HCA (BSA labelled with 7-hydroxycoumarin-4-acetic acid) by stopped-flow methods. Fatty acid–albumin complexes of a range of natural fatty acid types and albumin molecules (donors) were mixed with three fatty acid-binding acceptor proteins. Dissociation of fatty acids from the donor was monitored by either the time course of donor fluorescence/absorbance or the time course of acceptor fluorescence. The results of these measurements indicate that fatty acid dissociation from BSA as well as BSA-HCA is well described by a single exponential function over the entire range of fatty acid/albumin molar ratios used in these measurements, from 0.5:1 to 6:1. The observed rate constants (k_{obs}) for the dissociation of each fatty acid type reveal little or no dependence on

the initial fatty acid/albumin ratio. However, dissociation rates were dependent upon the type of fatty acid. In the case of native BSA with an initial fatty acid/BSA molar ratio of 3:1, the order of k_{obs} values was stearic acid (1.5 s^{-1}) < oleic acid < palmitic acid $\cong$ linoleic acid < arachidonic acid (8 s^{-1}) at 37°C . The corresponding values for BSA-HCA were about half the values for BSA. The results of this study show that the rate of fatty acid dissociation from native BSA is more than 10-fold faster than reported previously and that the off-rate constants for the five primary fatty acid-binding sites differ by less than a factor of 2. We conclude that for reported rates of fatty acid transport across cell membranes, dissociation of fatty acids from the fatty acid–BSA complexes used in the transport studies should not be rate-limiting.

Key words: ADIFAB, fatty acid-binding protein, fatty acid probe, fluorescence recording, 7-hydroxycoumarin.

INTRODUCTION

Serum albumin binds a variety of hydrophobic compounds, including non-esterified long-chain fatty acids (hereon referred to just as fatty acids), for which it serves as the principal transport protein in the plasma compartment [1]. Utilization of fatty acids for energy metabolism requires the rapid transport of fatty acids between serum and cells, and consequently the entire complement of serum fatty acids is turned over within about 2 min [2]. Albumin is central to this process because long-chain fatty acids (≥ 14 carbons) are highly insoluble in the aqueous phase [3,4]. Direct membrane interactions of fatty acid–albumin complexes have been implicated in the cellular uptake of fatty acids [5–7]. However, it is generally accepted that a major part of the fatty acid transport across blood capillaries occurs through spontaneous dissociation of fatty acids from the albumin into the aqueous phase of the intravascular compartment, followed by fatty acid transfer to the apical surface of the endothelial plasma membrane by simple diffusion.

Although a number of studies deal with the kinetics of fatty acid transport from the plasma compartment [8–10], our current understanding of the actual mechanism(s) by which fatty acid is transported in cell-membrane systems is limited [11–13]. In numerous studies of fatty acid membrane transport, BSA has been employed as a fatty acid donor and acceptor protein. For

example, recent model studies include analysis of intermembrane fatty acid transfer [14,15], fatty acid diffusion across red-blood-cell membranes [16,17] and liposomal membranes [18], and cellular uptake of fatty acids [19]. It is critical for interpreting the results of such studies that the kinetic constants that characterize the fatty acid–BSA interaction be well understood. Previous measurements have indicated oleic acid (OA) dissociation from BSA within a time scale of $< 10 \text{ s}$ [20], and rate constants ranging from 0.04 s^{-1} up to about 0.16 s^{-1} have been reported for OA dissociation from BSA and human serum albumin (HSA) at 37°C [21–24]. More rapid rates were measured in a study [25] on the dissociation of palmitate (26 s^{-1}) and stearate (0.6 s^{-1}) from HSA that had been modified chemically with an anthraniloyl fluorophore [26]. To obtain more accurate rate constants for dissociation of a range of natural long-chain fatty acids from native BSA, we have carried out kinetic measurements by stopped-flow mixing of fatty acid–BSA complexes with several different fatty acid-binding acceptor proteins and monitored the dissociation process by fluorometry. Additionally, we have compared the fatty acid dissociation kinetics of BSA with that of the BSA-related fatty acid probe BSA-HCA (BSA–7-hydroxycoumarin-4-acetic acid complex) [27,28], prepared by chemical modification of the albumin with 7-hydroxycoumarin-4-acetic acid. The results with BSA as well as BSA-HCA are consistent with a rapid fatty acid dissociation process. The observed rate

Abbreviations used: BSA-HCA, BSA–7-hydroxycoumarin-4-acetic acid complex; FABP, fatty acid-binding protein; L-FABP, liver FABP; ADIFAB2, Leu⁷² → Ala-modified rat intestinal FABP labelled with acrylodan; HSA, human serum albumin; k_{obs} , observed rate constant; k_{on} , k_{off} , association and dissociation rate constants; ν , average number of fatty acid molecules bound/molecule of albumin; OA, oleic acid.

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constants were more than 10-fold higher than reported previously for BSA, and range from about 6 s^{-1} to 0.9 s^{-1} at 37°C for C_{14} – C_{18} fatty acids. The dissociation time course is well described within the limits of experimental error by a single exponential function over the entire range of fatty acid/BSA molar ratios tested, from 0.5:1 to 6:1. The results are discussed in relation to the fatty acid-binding characteristics of the albumin molecule and the use of fatty acid–BSA complexes to buffer the aqueous-phase concentration of free fatty acids in transport studies.

EXPERIMENTAL

Materials

Fatty acid-free BSA was obtained from Boehringer Mannheim (Mannheim, Germany) and ICN (Costa Mesa, CA, U.S.A.). Fatty acids (98–99% purity) were from Sigma (St Louis, MO, U.S.A.) and fatty acid sodium salts were from Nu Chek Prep (Elysian, MN, U.S.A.). Absorbance and fluorescence measurements were done using buffers composed of 150 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4 and 20 mM Hepes (buffer A) or 150 mM NaCl and 20 mM Hepes (buffer B), both adjusted to pH 7.4. Stock solutions of fatty acids (20 mM) in ethanol and fatty acid sodium salt (10 mM) in 10 mM NaOH were stored at -20°C under nitrogen or argon. Recombinant rat liver fatty acid-binding protein (L-FABP) was prepared as described in [29]. The fatty acid probe ADIFAB2 (Leu⁷² → Ala-modified rat intestinal FABP labelled with acrylodan) was prepared and used as described in [3,30], and is available from FFA Sciences (San Diego, CA, U.S.A.). The fatty acid probe BSA-HCA was prepared by coupling 7-hydroxycoumarin-4-acetic acid to fatty acid-free BSA using methods described previously [27]. The degree of modification was 3.8 mol of 7-hydroxycoumarin-4-acetic acid/mol of BSA.

Fatty acid–albumin complexes

Complexes of fatty acid with BSA and BSA-HCA were prepared by serial addition at 37°C of $10\text{ }\mu\text{l}$ aliquots of fatty acid stock solutions in ethanol or fatty acid sodium salt in NaOH to fatty acid-free albumin ($100\text{ }\mu\text{M}$) in buffer A to the desired fatty acid/albumin molar ratios (between 0.5:1 and 6:1). The stock solutions were kept at 4°C and used within 1 week. When the complexes were used in the stopped-flow experiments, the final concentrations of ethanol and NaOH after mixing did not exceed 0.1% and $6\text{ }\mu\text{M}$, respectively. Control experiments confirmed that these concentrations had no significant effect on the recorded fatty acid dissociation time course. This was verified by measuring rates for an OA–BSA–HCA complex (where the average number of fatty acid molecules bound/molecule of albumin, ν , is 3.5) prepared from OA sodium salt as a function of increasing ethanol concentration (0–0.2%, v/v). In addition, equivalent stopped-flow recordings were obtained with complexes prepared from fatty acids in ethanol and fatty acid sodium salt in NaOH, confirming that the dissociation kinetics are independent of the method used to prepare the complexes. All dissociation data in the present report were from experiments done with the sodium salt of the fatty acid.

Stopped-flow methods

Stopped-flow absorbance measurements were done with reactant volumes of 0.4 ml using a Shimadzu spectrophotometer (model UV-3000) equipped with a rapid-mixing attachment. The dual-wavelength mode was used to minimize errors caused by changes in light scattering. The measuring and reference wavelengths were set at 375 and 420 nm, respectively. Absorbance readings

were taken every 2–20 ms and transferred to a data file consisting of 1000 data points. Instrument dead-time was estimated to about 10 ms. Stopped-flow fluorescence measurements were done using a KinTek Instrument (State College, PA, U.S.A.) with a dead-time of $< 2\text{ ms}$ and reactant volumes of 0.1 ml, as described previously [30]. For BSA-tryptophan measurements the excitation wavelength was 290 nm and emission was measured with a 20 nm band-width filter centred at 343 nm. For BSA-HCA, excitation was at 375 nm and emission was at 450 nm, and for ADIFAB2, fluorescence intensities were measured using two photomultipliers with filters at 440 and 550 nm. For each condition at least five scans of about 500 data points each were acquired and averaged.

Rate constants for the dissociation of fatty acids from BSA

Fatty acid dissociation from BSA was determined using three different methods. In all three methods, the time course of a fluorescence signal was measured following stopped-flow mixing of fatty acid–BSA complexes with one of three different fatty acid-binding acceptor molecules: L-FABP, ADIFAB2 and BSA-HCA. Previous studies have demonstrated that tryptophan fluorescence in BSA is quenched upon binding fatty acid [31]. We therefore monitored the increase in tryptophan fluorescence as fatty acid dissociated from BSA in the presence of the L-FABP acceptor (L-FABP has no tryptophan). Stopped-flow traces were analysed using one- and two-component exponential functions and in virtually all cases no significant improvement in fit quality was obtained with a two-component fit. Results were characterized therefore by the observed rate constant (k_{obs}) calculated by fitting the time course with a single-exponential function. In accordance with [31], calibration curves giving the relationship between BSA-tryptophan fluorescence intensity and ν were close to linear up to $\nu = 4$. In dissociation experiments carried out at $\nu > 4$ there may have been an error in the calculated rate constants due to the deviations from linearity in the calibration curves. Therefore, corrections were made using appropriate calibration curves in combination with a transformation of the stopped-flow readings into actual fatty acid concentrations. It was found that the corrected and non-corrected rate constants differed by about 15% at $\nu = 6$. For ADIFAB2 as the acceptor, the increase in the 550 nm/440 nm emission ratio of ADIFAB2 was monitored as fatty acid moved from BSA to ADIFAB2. The k_{obs} values for BSA were determined by analysing these time courses in terms of a kinetic model that accounts for fatty acid dissociation and binding to BSA and ADIFAB2, using the program MLAB as described previously for fatty acid-binding proteins (FABPs) [30]. With BSA-HCA as the acceptor, k_{obs} values were used to provide limits for the rate of fatty acid dissociation from BSA. The techniques using ADIFAB2 and BSA-HCA as fatty acid acceptors are independent of the non-linearity of BSA-tryptophan fluorescence.

Rate constants for the dissociation of fatty acids from BSA-HCA

The dissociation of fatty acids from BSA-HCA was determined by monitoring the time course of the BSA-HCA absorbance increase or fluorescence increase following stopped-flow mixing of fatty acid–BSA–HCA complexes with fatty acid-free BSA. Absorbance measurements at 375 nm were done by mixing 3–15 μM BSA-HCA with 18–760 μM fatty acid-free BSA and the fatty acid/BSA-HCA molar ratio was between 0.5:1 and 5:1. Fluorescence measurements (emission at 450 nm with excitation at 375 nm) were done by mixing 1 μM BSA-HCA with 10 μM fatty acid-free BSA. As for BSA, the k_{obs} values for BSA-HCA were determined by analysing the stopped-flow time courses

using a single exponential function. In dissociation experiments carried out at fatty acid–BSA–HCA ratios exceeding $\nu = 4$, corrections were made for the deviations from linearity in the calibration curves, as in [27,32]. The corrected and non-corrected rate constants differed by 5–7% at $\nu = 4.7$.

RESULTS

Kinetics of OA dissociation from BSA

In initial experiments, time courses for OA dissociation from BSA were determined following stopped-flow mixing of OA–BSA complex with excess fatty acid-binding acceptor protein, L-FABP, BSA-HCA or ADIFAB2. The OA transfer was monitored by three different methods: (i) from the intensity increase in BSA-tryptophan fluorescence that occurs as the OA dissociates from the OA–BSA complex; (ii) from the decrease in 7-hydroxycoumarin fluorescence as OA binds to BSA-HCA and (iii) from the change in acrylodan fluorescence as OA binds to ADIFAB2. A typical set of stopped-flow time courses is shown in Figure 1. The time courses of BSA-tryptophan fluorescence were well described by a single exponential fit (Figure 1A). The k_{obs} values derived from the time courses were independent of the L-FABP concentration at L-FABP/BSA molar ratios of 5:1 and 10:1. Rate constants also revealed little or no dependence

Figure 1 Fluorescence time courses for the dissociation of OA from BSA to three different acceptors

Measurements were done by stopped-flow mixing of OA–BSA complexes with L-FABP, BSA-HCA and ADIFAB2 at 37 °C. Changes in fluorescence intensities of either the donor BSA or the acceptor molecules were used to monitor dissociation. (A) Tryptophan fluorescence of OA–BSA complexes after mixing with excess L-FABP. Concentrations after mixing were [BSA] = 0.5 μM , [L-FABP] = 5 μM and $\nu = 0.5, 1.5$ and 6 as indicated. Single exponential fits to these data yielded k_{obs} values of 1.8, 3.6 and 2.6 s^{-1} , respectively. (B) 7-Hydroxycoumarin fluorescence of BSA-HCA after mixing OA–BSA complex with excess BSA-HCA. Concentrations after mixing were [BSA] = 0.5 μM , [BSA-HCA] = 2 μM and $\nu = 3.0$. A single exponential fit yielded $k_{\text{obs}} = 2.1 \text{ s}^{-1}$. (C) Acrylodan fluorescence of ADIFAB2 after mixing OA–BSA complex with excess ADIFAB2. Concentrations after mixing were [BSA] = 0.5 μM , [ADIFAB] = 1 μM and $\nu = 4.0$. Analysis of the time course using the program MLAB [30] yielded $k_{\text{obs}} = 1.8 \text{ s}^{-1}$.

Figure 2 Dependence of k_{obs} on the OA/BSA molar ratio

Time courses of OA dissociation from BSA were recorded at 37 °C by fluorescence detection after stopped-flow mixing of OA–BSA complex ($\nu = 0.5$ –6) with excess L-FABP and BSA-HCA as fatty acid acceptor. Concentrations after mixing were 0.25–1 μM for [BSA], 5 or 10 μM for [L-FABP] and 2–8 μM for [BSA-HCA]. Values for k_{obs} were determined from single exponential fits to the transfer time courses. Results shown are means $\pm$ S.D. The values measured with L-FABP at $\nu = 6$ were corrected for the deviations from linearity in the calibration curves. The linear fit revealed no significant dependence of k_{obs} on the OA/BSA ratio.

Table 1 Rates of FA dissociation from BSA

Values for k_{obs} were determined from single exponential fits to the time courses of BSA-tryptophan fluorescence recorded after stopped-flow mixing of fatty acid–BSA complex ($\nu = 3.0$) with L-FABP. Fatty acid–BSA-HCA complexes were prepared from fatty acid sodium salt. Final concentrations after mixing were: [BSA] = 0.5 μM and [L-FABP] = 5 μM . Values are means $\pm$ S.D. from three or four separate datasets for each fatty acid type.

Fatty acid	k_{obs} (s^{-1})	
	37 °C	22 °C
Palmitic acid ($\text{C}_{16:0}$)	6.9 ± 0.6	1.9 ± 0.1
Stearic acid ($\text{C}_{18:0}$)	1.5 ± 0.06	0.7 ± 0.02
Oleic acid ($\text{C}_{18:1}$)	3.3 ± 0.9	1.1 ± 0.2
Linoleic acid ($\text{C}_{18:2}$)	6.8 ± 0.6	2.3 ± 0.07
Linolenic acid ($\text{C}_{18:3}$)	4.2 ± 0.3	2.9 ± 0.2
Arachidonic acid ($\text{C}_{20:4}$)	8.1 ± 2.0	3.2 ± 0.2

on the initial OA/BSA ratio, yielding at 37 °C values for k_{obs} from 1.8 s^{-1} for the smallest initial OA/BSA molar ratio (0.5:1) to 2.6 s^{-1} for the largest ratio (6:1; Figure 1A). Single exponential time courses were also obtained for OA transfer from OA–BSA complex using BSA-HCA as an acceptor, yielding $k_{\text{obs}} = 2.1 \text{ s}^{-1}$ (Figure 1B). Similar rates were obtained with BSA/BSA-HCA molar ratios of 1:2 and 1:10 and k_{obs} did not vary significantly, from 2.0 ± 0.4 to $2.7 \pm 0.2 \text{ s}^{-1}$ (means $\pm$ S.D.), with increasing initial OA/BSA molar ratios from 0.5:1 to 6:1 (Figure 2). Although BSA-HCA has sufficient fatty acid binding affinity to provide an efficient sink for the dissociation of OA from BSA, analysis of the dissociation time courses is more complex with this probe than with a probe having a single binding site [33]. For this reason we also measured the dissociation time course of OA–BSA complexes using ADIFAB2, the high-affinity version of ADIFAB, as an acceptor. Results of these measurements yielded $k_{\text{obs}} = 1.8 \text{ s}^{-1}$, consistent with the other two methods (Figure 1C). Thus the k_{obs} values characterizing the fatty acid

Figure 3 Light absorption and fluorescence time courses for the dissociation of OA from BSA-HCA

Measurements were done by stopped-flow mixing OA-BSA-HCA complexes with fatty acid-free BSA at 37 °C. **(A)** Light absorption at 375 nm with reference wavelength at 420 nm. Concentrations after mixing were [BSA-HCA] = 3.43 μM ($\nu = 4.7$) and [fatty acid-free BSA] = 37.8 μM . A single exponential fit to this time course yielded $k_{\text{obs}} = 1.6 \text{ s}^{-1}$. **(B)** Fluorescence emission at 450 nm. Concentrations after mixing were [BSA-HCA] = 0.5 μM ($\nu = 1.0$) and [fatty acid-free BSA] = 5 μM . A single exponential fit yielded $k_{\text{obs}} = 1.4 \text{ s}^{-1}$.

dissociation process are independent of the type of fatty acid acceptor protein used and independent of whether the process is recorded by a signal from the BSA or from the acceptor. All of these results validate our stopped-flow conditions for recording fatty acid dissociation kinetics.

Variation of fatty acid-BSA dissociation rate with fatty acid type

L-FABP was chosen as the fatty acid acceptor in subsequent stopped-flow fluorescence experiments carried out to measure the dependence of the rate of fatty acid-BSA complex dissociation on fatty acid type. Measurements of k_{obs} were performed at a high (10:1) molar ratio of acceptor L-FABP to donor BSA. For all fatty acids tested in the C_{16} to C_{20} chain-length range, the dissociation time courses were well described by single exponential functions within the experimental error of the measurements. Data obtained at 22 and 37 °C for complexes with a fatty acid/BSA molar ratio of 3:1 are compiled in Table 1. As these results indicate, the k_{obs} values which range between approx. 0.7 and 8 s^{-1} decrease with fatty acid chain length ($\text{C}_{16:0}$ – $\text{C}_{18:0}$) and increase with the degree of fatty acid unsaturation ($\text{C}_{18:0}$ – $\text{C}_{18:2}$).

Kinetics of OA dissociation from BSA-HCA

We have shown previously that BSA-HCA retains a fatty acid-binding affinity not very different from that of native BSA [27,28]

and this property makes it a potential fatty acid probe allowing for fluorescence detection of the very low aqueous-phase concentrations of free fatty acids that are biologically relevant [32]. Data on the kinetics of fatty acid interaction with BSA-HCA are essential to the application of the probe in studies of fatty acid transport across cell membranes and it was therefore included in the present study. Time courses for the dissociation of OA from BSA-HCA were determined directly by measuring either the increase in light absorption or the increase in fluorescence emission intensity of OA-BSA-HCA complexes following stopped-flow mixing with an excess of fatty acid-free BSA. As can be seen in Figure 3, the time courses recorded with both methods were well described by a single exponential function. At a fixed BSA-HCA concentration the measured k_{obs} values did not vary significantly, from $1.2 \pm 0.3 \text{ s}^{-1}$ to $1.5 \pm 0.2 \text{ s}^{-1}$ (means $\pm$ S.D.) at 37 °C, as the initial OA/BSA-HCA molar ratio increased from 0.5:1 to 4.7:1 (Figure 4). The k_{obs} values exhibited no significant change when the measurements were performed using a range of acceptor concentrations (9–380 μM) corresponding to molar ratios of acceptor BSA/donor BSA-HCA of between 2.6:1 and 112:1 (Figure 5). This result is consistent with fatty acid exchange between albumin molecules by diffusion through the aqueous phase, as found previously for fatty acid transfer between BSA and liposomes [15,22]. Thus we find no evidence for a collisional fatty acid exchange at albumin concentrations that are close to physiological relevance.

Figure 4 Dependence of k_{obs} on the OA/BSA-HCA molar ratio

Time courses of OA dissociation from BSA-HCA were recorded at 20 and 37 °C by 375 nm light-absorption detection after stopped-flow mixing of OA-BSA-HCA complex ($\nu = 0.5\text{--}4.7$) with excess fatty acid-free BSA as fatty acid acceptor. Concentrations after mixing were [BSA-HCA] = 3.4 μM and [fatty acid-free BSA] = 38 μM . Values for k_{obs} were determined from single exponential fits to the transfer time courses and results are shown as means $\pm$ S.D. ($n = 3$). The linear fit revealed no significant dependence of k_{obs} on the OA/BSA-HCA ratio.

Variation of fatty acid-BSA-HCA dissociation rate with fatty acid type

In stopped-flow experiments using fatty acid-free BSA as a fatty acid acceptor, the dissociation time courses of fatty acid-BSA-HCA complexes were well described by a single exponential fit for all fatty acid types tested. As in the measurements of fatty acid-BSA dissociation, k_{obs} values were determined at a 10:1 molar ratio of acceptor/donor. Values obtained at 20 and 37 °C with fatty acids in the C_{16} to C_{18} chain-length range are compiled

Figure 5 Acceptor concentration dependence of k_{obs} for OA dissociation from BSA-HCA

The increase in 375 nm light absorption of OA-BSA-HCA complex ($\nu = 4.2$) was recorded at 20 °C after stopped-flow mixing with increasing concentrations of fatty acid-free BSA. Final concentrations after mixing were [BSA-HCA] = 3.4 μM and [fatty acid-free BSA] = 9.5–378 μM . Values for k_{obs} were determined as for Figure 4. The linear fit revealed no significant dependence of k_{obs} on the OA/BSA ratio.

Table 2 Rate and equilibrium constants for fatty acid interaction with BSA-HCA

Values for k_{obs} were determined from single exponential fits to the time courses of BSA-HCA absorbance recorded after stopped-flow mixing of fatty acid-BSA-HCA complex ($\nu = 3.7\text{--}4.7$) with excess fatty acid-free BSA. Final concentrations after mixing were: [BSA] = 38 μM and [BSA-HCA] = 3.4 μM . Fatty acid-BSA-HCA complexes were prepared from fatty acid sodium salt. Values are means $\pm$ S.D. from three or four separate datasets for each fatty acid type. K_{d} values are from [28] and values of k_{on} (the association rate constant) were calculated according to the relationship $k_{\text{on}} = k_{\text{off}}/K_{\text{d}}$, with $k_{\text{off}} = k_{\text{obs}}$ (where k_{off} is the dissociation rate constant).

Fatty acid	k_{obs} (s^{-1})		K_{d} (nM)	k_{on} ($10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$)
	37 °C	20 °C	20 °C	20 °C
Myristic acid ($\text{C}_{14:0}$)	5.77 ± 0.65	1.73 ± 0.17	459	0.38
Palmitic acid ($\text{C}_{16:0}$)	3.46 ± 0.33	0.8 ± 0.09	104	0.77
Stearic acid ($\text{C}_{18:0}$)	0.89 ± 0.06	0.31 ± 0.06	14	2.2
Oleic acid ($\text{C}_{18:1}$)	1.55 ± 0.2	0.42 ± 0.07	22	1.9
Linoleic acid ($\text{C}_{18:2}$)	1.76 ± 0.16	0.68 ± 0.02	40	1.7

in Table 2. The values range between approx. 0.3 and 6 s^{-1} , and as in the case of underivatized BSA they decrease with fatty acid chain length ($\text{C}_{14:0}\text{--}\text{C}_{18:0}$) and increase with the degree of unsaturation ($\text{C}_{18:0}\text{--}\text{C}_{18:2}$).

DISCUSSION

The objective of this study was to determine the rate constants for fatty acid dissociation from BSA in order to better understand the mechanisms for membrane transport of fatty acid. For the physiologically important fatty acid types tested, the dissociation time courses are well described by a single exponential function. The k_{obs} values determined at 37 °C were found to range from about 1.5 s^{-1} ($\text{C}_{18:0}$) to 8 s^{-1} ($\text{C}_{20:4}$).

Using stopped-flow fluorometry, Massey et al. [25] reported rapid first-order kinetics for stearate (0.6 s^{-1}) and palmitate (26 s^{-1}) dissociation from HSA chemically modified with an anthraniloyl fluorophore [26]. However, the rate constants reported previously for fatty acid dissociation from native albumins are more than an order of magnitude lower than our present data and furthermore vary widely between the different experimental systems. For example, in the case of OA, rate constants that range from 0.04 to 0.16 s^{-1} were reported [21–24]. In the studies in which bound and free fatty acids were physically separated [21,23], the temporal resolution ($> 5 \text{ s}$) would not have been sufficient to resolve a dissociation process with a rate constant of 2–3 s^{-1} , as observed for OA in the present study. Measurements involving spectroscopic and dielectric changes were done using stopped-flow mixing and should therefore possess the requisite temporal resolution [22,24]. However, these measurements yield low rates as well with a rate constant of about 0.04 s^{-1} . Although in the studies of [22], rate constants were determined from measurements of BSA-tryptophan fluorescence, BSA was used as an acceptor for OA transfer from lipid vesicles. In this configuration, fatty acid dissociation from the vesicles dominates the time course, making it difficult to extract accurately the fatty acid dissociation from BSA. Finally, in studies where fatty acid dissociation from BSA was deduced from the measured rates of fatty acid diffusion across cell membranes, the reported dissociation rate constants for palmitic acid ranged from 0.0026 s^{-1} (0 °C) [34] to 0.27 s^{-1} (37 °C) [9].

The accurate determination of dissociation rate constants from the measurement of transfer rates between donors and acceptors depends upon the rate constants, affinities and concentrations of the donor and acceptor populations [15,35]. In the

present study, equation A11 from [35] predicts less than a 10% difference between dissociation and transfer rates, using the affinities and rates of the L-FABP and BSA-HCA acceptors at a 10:1 molar ratio of acceptor/BSA. Moreover, using ADIFAB2 as an acceptor yields the k_{off} (the dissociation rate constant) directly, because the complete set of rate equations was used in the analysis of the ADIFAB2 results. We speculate that the low and variable rates observed in the previous studies may be due partly to the use of different fatty acid acceptor/donor ratios and that the measured rate constants are more reflective of fatty acid diffusion times between donor albumin and the acceptor molecules than the actual dissociation step from the donor.

Equilibrium binding studies with BSA as well as other serum albumins reveal that the albumin molecule contains multiple fatty acid-binding sites with a range of affinities [36–40]. Binding isotherms are compatible with a model having two or three high-affinity sites that appear to be close to equivalent within the uncertainty of the data analysis [4,41,42], in addition to several low-affinity sites. This is consistent with ^{13}C -NMR studies on BSA indicating two classes of affinity: higher ones for the sites populated at fatty acid/BSA ratios of $\leq 3:1$ and lower affinities at higher ratios [43,44]. Furthermore, the recently resolved X-ray crystallographic structure of HSA in complex with fatty acids [45,46] reveals at least five primary fatty acid-binding sites throughout the albumin molecule, each site with distinct chemical environments and conformations for the fatty acid ligand molecule. Nevertheless, the k_{obs} values measured at the smallest initial fatty acid/BSA ratios (0.5:1), where high-affinity sites are populated, were not significantly different from those at larger ratios. This result indicates a similar dissociation process for the five primary fatty acid-binding sites of BSA. The finding that the time course for dissociation can be fitted by a single exponential over the entire range of initial fatty acid/BSA molar ratios from 0.5 to 6 is consistent with this conclusion, although two components whose rates differ by less than 2-fold cannot be resolved from the stopped-flow data. We speculate that exchange of fatty acids between binding sites on the surface of the albumin molecule on a timescale that is faster (< 0.5 s) than the kinetic measurements but slower than the NMR timescale (> 5 –40 ms) [43,44] might result in homogeneous behaviour.

Similar to native BSA, the fatty acid dissociation time courses for the fatty acid probe BSA-HCA are first order up to a fatty acid/BSA-HCA molar ratio of about 5:1. However, as can be seen from a comparison of the data in Tables 1 and 2, the k_{obs} values are generally about half the values for BSA. Fatty acid-binding isotherms for BSA-HCA as determined by direct fluorescence titration are, within the experimental uncertainty of this type of experiment, well represented by a binding model with four or five equivalent high-affinity sites [28]. Using the previously published K_{d} values and the simplifying assumption that k_{off} for all sites is equal to the k_{obs} values measured in the present work, we can estimate k_{on} (the association rate constant) for the fatty acid association with BSA-HCA. Data for the different fatty acid types are included in Table 2. Interestingly, the calculated k_{on} values $[(0.4\text{--}2) \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}]$ are in the same range as the values reported previously for fatty acid association with FABP [30,33]. In studies that are in progress we are using the fatty acid probe ADIFAB to monitor OA binding to native BSA. Analysis of the binding isotherms according to a binding model with five equivalent sites yields a K_{d} value of 14 nM at 37 °C. The corresponding value for OA binding to BSA-HCA at 37 °C is 44 nM. Using these data and the assumptions in Table 2, the k_{on} values for OA association with BSA and BSA-HCA at 37 °C are estimated to be 24×10^7 and $3.5 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$, respectively. This result shows that the four 7-hydroxycoumarin molecules present

in the BSA-HCA probe not only reduce the k_{off} but reduce the k_{on} value for the fatty acid–albumin interaction as well. One or more of the 7-hydroxycoumarin labels thus appear to be positioned to partially block fatty acid binding or to reduce the conformational flexibility of the albumin molecule. This is reminiscent of ADIFAB, where the acrylodan label reduces k_{on} about 8-fold relative to unlabelled FABP [30].

The mechanism by which fatty acids are transported across cell membranes is a subject of intense debate [11–13,19]. Three kinetic steps are generally considered to characterize the membrane-transport process in cellular fatty acid uptake: (i) binding of fatty acids from the aqueous phase to the outer surface of the membrane; (ii) flip-flop of the fatty acids from the outer to inner surface and (iii) dissociation of the fatty acids from the inner surface. The focus of this debate has been whether flip-flop or dissociation are rate-limiting. It is generally assumed that the binding step is rapid and most studies have been done using fatty acid–BSA complexes to buffer the aqueous concentration of free fatty acids and to deliver fatty acids to the membranes. To determine whether transfer of fatty acids from BSA into the aqueous phase is rate-limiting for the membrane-transport process in these studies, rate constants for fatty acid dissociation from BSA and the membrane, as well as parameters describing the relative amounts of BSA and membrane, must be known [35]. Up to now, most results suggest that transport of long-chain fatty acids across cell membranes occurs with rate constants of $\leq 1 \text{ s}^{-1}$ [13,19]. This is about the same order as the dissociation rate constants observed in the present study for BSA (Table 1), raising the possibility that transfer from BSA might be rate-limiting, especially if cellular transport rates were significantly greater than 1 s^{-1} in the presence of fatty acid-transport proteins. However, if the amount of fatty acids initially bound to BSA is greater than the amount transferred to the cells or, in other words, if the fatty acid–BSA complex adequately buffers the unbound non-esterified fatty acid concentration, then the time to reach equilibrium for fatty acid transfer from BSA to the membranes will be shorter than $(k_{\text{off}})^{-1}$. For example, in our study of fatty acid transport across human erythrocytes [17], the time for transfer of OA from BSA to the membranes was estimated to be < 130 ms. Thus for appropriate conditions in transport studies, fatty acid transfer from BSA will not be rate-limiting for measurements of fatty acid transport across membranes unless cells are identified with transport rates of $> 10 \text{ s}^{-1}$.

This work was supported by a grant from the Novo Nordisk Foundation (to E.J.F.D.) and by grant DK58762 from the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health (to A.M.K.). The expert technical assistance of Mrs Birthe Taanning Nyström is gratefully acknowledged.

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Received 29 October 2001/25 January 2002; accepted 21 February 2002