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Monoglyceride-Protein Interaction

The Binding of Monoolein to Native Human Serum Albumin

E. O. ARVIDSSON and P. BELFRAGE

Division of Physiological Chemistry, Chemical Center, University of Lund, Box 740, S-220 07 Lund 7, Sweden

The binding of monoolein to native human serum albumin has been studied using (^{3}H) glycerol-labeled monoolein at pH 7.5, 37 and 25°C.

The free energy for the binding was shown to originate from an entropy change. This is discussed in terms of a model previously postulated for the binding of alkyl anions to serum albumin.

When evaluating the biological effect of a small molecule *in vivo* or *in vitro* the total concentration may be completely misleading: most of it may be bound to some protein and only a small fraction may be unbound or free. Thus it is important to know the relative distribution of a compound between the free and the bound form, since only one of these two forms may be able to reach its target. In some instances, *e.g.* in coenzyme-enzyme interactions, the complex constitutes the biologically active part.

A quantitative descripton of the interactions between small molecules and macromolecules such as proteins is necessary in order to understand the nature of these interactions. Recent reviews are given by Rosenberg and Klotz [1] and by Arvidsson.[2]

The binding theory hitherto most commonly used may be termed the "site-class theory". Its main feature is the division of the binding sites into definite classes. No interactions between the bound molecules other than electrostatic are considered. Thus a chloride ion and a short-chain and a long-chain carboxylate ion are treated in the same way, although their isotherms are very different. In a monograph (Arvidsson [2]) a novel binding theory has been developed.

The new binding theory assumes that the anion binding sites are all the positively charged groups of a protein and further assumes that all sites have the same binding strength on the average. Statistical mechanics was used in the derivations of the binding isotherms. Interactions are permitted among bound molecules and between a bound molecule and the neighbour loci.

Also interactions among the binding loci (positively charged) and negative carboxylate residues on the protein surface are permitted (=salt bonding or salt bridges). The "abnormal" binding (and titration) properties of native serum albumin have been discussed in terms of locking of its polar groups into non-equilibrium positions by the S—S bonds.

The complete binding isotherm is perhaps most easily described in terms of three "types" of binding. Since all types can occur with all loci, these types have nothing in common with the binding classes mentioned above:

Type I. Nearest neighbour sites empty, large polar contribution. Hydrophobic part.

Type II. Nearest neighbour sites filled (or site involved in "salt bonding"), small polar contribution. Hydrophobic part as in I. Neighbour interactions may cause a 1st order phase transition for long-chain alkyl compounds.

Type III. Binding of micellar type, *i.e.* polar heads not at all or badly localized. Type III binding is only present with molecules having a hydrocarbon part: the hydrophobic material of the molecules bound according to I and II furnishing "new sites" in III.

One of our aims with this investigation was to test this model.

Since monoolein bound to human serum albumin had been used as a substrate source in a study of a monoglyceride-splitting enzyme (Belfrage [3]) another aim of the present investigation was to obtain more information on the nature of this monoolein-albumin-complex.

MATERIALS AND METHODS

Preparation and purification of labeled monoolein: 2-n-(^{3}H) glycerol was obtained from the Radiochemical Centre, Amersham, England and was used without further purification. Oleic acid was obtained from Fluka AG, Buchs, Switzerland. The purity of the oleic acid was tested by gas-liquid chromatography. More than 99 % of the total fatty acid mass was found in the oleic acid peak. Oleyl chloride was prepared from this oleic acid as reported previously from this department (Borgström and Jordan [4]).

(^{3}H) Glycerol-labeled glycerides were then prepared from the (^{3}H) glycerol and the oleyl chloride essentially as described for labeled glycerides previously.[4] Monoolein was isolated with preparative thin-layer chromatography on silica gel G (Merck, Darmstadt, W. Germany) using a heptane-diethyl ether (80:20 v/v) solvent system. The monoolein was identified by comparing with a purified monoolein standard and was then eluted from the silicic acid with methanol. The monoolein was purified in the same way once more using the same solvent system. Thin-layer chromatography revealed that more than 98.5 % of the (^{3}H)-radioactivity was found in the monoglyceride spot.

Purification of unlabeled monoolein: Monoolein was obtained from Eastman Kodak Company, Rochester, U.S.A. and was purified twice with preparative thin-layer chromatography. It was shown with gas-liquid chromatography that more than 98 % of the fatty acid mass was oleic acid.

Human serum albumin (HSA) was a gift from AB Kabi, Stockholm, Sweden (amorphous, 99 % albumin in free electrophoresis). It was dialyzed and defatted as described before.[2]

The protein concentration was estimated by determining the absorption at 280 mμ. $E_{280}(1\%)$ was taken to 5.3.[5]

The buffer was a Tris-HCl buffer of pH 7.5 and $\Gamma/2=0.15$. The buffer salt contribution to the ionic strength was 0.05, the remaining 0.10 arising from sodium chloride.

Estimation of protein binding. Principles and experimental details of the present method are discussed extensively in Ref. 2 and only a brief description is given below: Two buffer/water phases, I and III, are separated by a heptane phase II. III also contains

protein. The monoglyceride distributes itself until its chemical potential is the same in all the phases I, II, and III. The concentration of free, unbound monoglyceride, c, is then found in I. The total concentration in III equals the sum of the free and the bound monoglyceride. From this the mean binding, $\overline{N}$, in moles monoglyceride per mole protein is easily obtained, since the protein concentration is known.

The binding was determined after equilibration for 4 to 5 days at 25 and at 37°C. The concentration in phase I was obtained after extraction of an aliquot, 23 ml, and determination of the radioactivity in a liquid scintillation counter. The radioactivity in phase III could be measured without extraction, 0.5 ml being sufficient. The protein concentration did not exceed 10^{-4} M.

RESULTS AND DISCUSSION

Fig. 1 shows the mean binding $\overline{N}$, *i.e.* number of monoolein molecules bound on a serum albumin molecule averaged over the whole ensemble of molecules in the system (sample), as a function of the logarithm of the concentration of free monoolein. Log c can be regarded proportional to the chemical potential at a given temperature.

The assay system in Ref. 3 contained human serum albumin, 50 mg, and monoolein, 5 μmoles, in a volume of 0.5 ml. If we assume provisionally that all the monoolein is bound this corresponds to an $\overline{N}=7$. Fig. 1 shows that this value requires a free monoolein concentration, c, of approximately 10^{-6} M. Since the total monoolein concentration is 10^{-2} M it can be concluded that only a 1/10 000 of the monoolein is in an un-bound form.

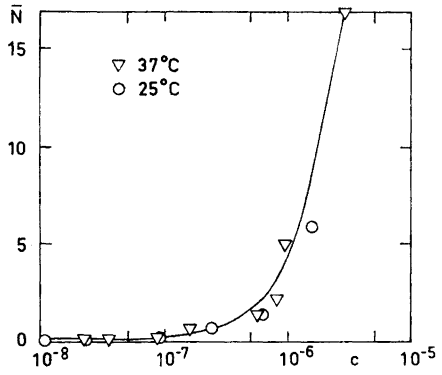

Fig. 1. The mean binding, $\overline{N}$, of monoolein to native human serum albumin as a function of the concentration of free monoolein, c, at 37°C and 25°C. (For further details, see text.)

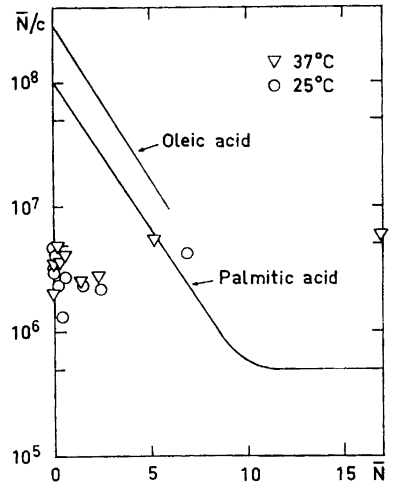

Fig. 2. $\overline{N}/c$ *vs.* $\overline{N}$ for the binding of monoolein to native human serum albumin at 37°C and 25°C (for further details, see text). Also shown in the figure: the binding isotherm for palmitic acid (from Ref. 2) and oleic acid (from Ref. 6).

Fig. 2 shows the same values in another plot: log $\bar{N}/c$ as a function of $\bar{N}$. This last representation has some unique features which should make it the preferred one in binding studies: if the binding isotherm has the "Langmuir form"

$$\bar{N} = Mkc/(1+kc)$$

where M is the number of binding sites, $\bar{N}$ the mean binding, c the concentration (or activity) of free solute, and k is an association "constant", then the intercept on the ordinate is

$$\log (\bar{N}/c)_{\bar{N},c\to 0} = \log Mk^\circ$$

Here we assume that k is not independent of $\bar{N}$, *i.e.* $k=k^\circ f(\bar{N})$ and $f(0)=1$. In general, one can assume that the partition function obtained in a statistical mechanical treatment can be factorized: this means that log $f(\bar{N})$ can be written as a sum of such functions, which simplifies quantitative comparisons between isotherms for different compounds. A polar and a non-polar contribution may be assigned to the statistical-mechanical site partition function (corresponding to the association constant in the binding isotherm). The polar part can be described in terms of ion-pair formation for the binding of an anion. Previous studies [2] have shown that the polar contribution is weakened or vanishes if (a) the site is involved in a "salt bond" with a protein carboxyl group, or if (b) a neighbouring locus is occupied: if the weakening effect on the polar part from an occupied neighbouring site is small, the effect could be treated by the quasi-chemical approximation of nearest neighbour statistics; if the polar part vanishes, the nearest neighbours can be treated as "forbidden" sites. The latter situation was found to obtain in the case of fatty acid binding to serum albumin.

The non-polar part was found to be additive, $\Delta\Delta G^\circ$ per CH_2-group being 0.5 kcal. The critical concentration for micelle formation is dependent on the hydrocarbon chain length in a similar manner. This dependence can be developed quantitatively by means of statistical mechanics: a figure of 0.7 kcal was found for $\Delta\Delta G^\circ$ per CH_2-group. The difference, 0.2 kcal, can be discussed as an entropy effect, arising from a greater freedom to move for the hydrocarbon tail in a micellar state as compared to a surface adsorbed state. Furthermore, ΔH° for the binding of palmitic acid to serum albumin is about zero, and the change in ΔH°, $\Delta\Delta H^\circ$ per CH_2-group, in going to another fatty acid, is also zero or near to zero: this can be interpreted to indicate that the hydrocarbon chain is curled up in a bad solvent. The novel binding model discussed above, enables us to predict a binding isotherm for a monoglyceride: we expect that the postulated *type I* binding should vanish for a non-electrolyte and that the binding should be of *type II*, *i.e.* with a small polar contribution to the site partition function. (Actually, "small" means, that the polar interaction of a monoglyceride with a binding site should be of the same order of magnitude as the polar part of its interaction with the solvent molecules.) The result should be a flat curve in a log $(\bar{N}/c)$ *vs.* $\bar{N}$ plot with an $(\bar{N}/c)$-value approximating that for *type II* binding, *i.e.* the $(\bar{N}/c)$

value for oleic acid binding at $\bar{N}$ values of the order of 10 to 20. The oleic acid curve shown in Fig. 2 has been reconstructed from data given by Goodman.[6] From the palmitic acid curve, taken from Ref. 2, it is seen that $\bar{N}/c$ (oleic acid) $\simeq 2.5$ $\bar{N}/c$ (palmitic acid). $\bar{N}/c$ for palmitic acid binding of *type II* is about 5×10^{-5}. Thus the monoglyceride isotherm should be a "straight line", $\bar{N}/c \simeq 1.3 \times 10^{6}$. Actually, the experimental points do fit a straight line but at a somewhat higher $\bar{N}/c$ -value. The small difference, a factor of about 3 to 4 corresponding to 0.7 to 0.8 kcal, might arise from the glyceryl group, present in monoolein but not in oleic acid.

The free energy for the fatty acid binding to serum albumin arises from an entropic change, ΔH° for the binding process being zero in the temperature region 25—37°C. Since this entropic change can be explained as an effect of interaction between the hydrocarbon part of an alkyl compound and the solvent molecules ("hydrophobic bonding"; see chap. 3—8 in Ref. 2) we expect that this change should not be affected, if the carboxyl group is replaced by a glyceryl group. Therefore the monoglyceride binding should not be temperature dependent. Inspection of Fig. 2 confirms this assumption: the points for the two temperatures should fit the same curve (not drawn for sake of clarity) within experimental error.

In conclusion, it can be stated that the binding isotherm for monoolein binding to serum albumin is in support of the model for small molecule-protein interactions outlined above. (For a more detailed thermodynamic discussion reference must be made to Arvidsson [2]).

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