AN ANALYSIS OF THE BINDING OF 8-ANILINO-1-NAPHTHALENE-SULPHONATE TO SUB-MITOCHONDRIAL PARTICLES

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1. Introduction

The negatively charged fluorescent probe 8-anilino-1-naphthalene sulphonic acid (ANS) has been widely used as an indicator of the state of energisation of mitochondrial membranes [1–10]. The fluorescence yield of ANS bound to sub-mitochondrial particles is observed to increase, and that of ANS bound to whole mitochondria to decrease, on energisation.

These changes in fluorescence have been variously attributed to changes in the quantum yield of the probe, the extent of its binding, and a combination of both factors. It is now generally accepted that independent of any possible change in quantum yield a substantial change in the number of probe molecules bound to the membrane does take place on energisation. Direct binding studies demonstrating such changes, which are thought to reflect changes in charge distribution and structure of the membrane [11], have been carried out by several authors [2, 8, 10].

In this paper, we compare the results of such direct binding studies with results obtained by an indirect method based on measurements of the fluorescence of the bound probe. The two methods give different results. The reasons for these discrepancies are discussed and used as a basis for a re-interpretation of the earlier data.

2. Materials and methods

Heavy beef heart mitochondria were prepared by the method of Löw and Vallin [12], and from these EDTA particles were prepared by the method of Lee and Ernster [13]. The magnesium salt of ANS, obtained from Eastman Kodak, Ltd., was re-crystallised twice from hot aqueous solutions before use. The direct binding studies were carried out using the method described by Nordenbrand and Ernster [8]. The indirect studies were based on a method described by Brocklehurst et al. [3]. Details of the reaction media used are given in the relevant figure captions.

3. Results and discussion

3.1. *Direct binding studies*

The method adopted in these studies was essentially that of Nordenbrand and Ernster [8]. Suspensions of EDTA particles containing varying concentrations of ANS were energised by the addition of succinate. They were then cooled, the particles separated out by ultracentrifugation and the concentration of free probe in the supernatant determined by fluorescence measurements. Several methods of cooling were tested. The most efficient method was found to be by transfer of the suspension to thick walled test tubes pre-cooled to about −10°C in an ice–salt freezing mixture. Using this method the samples could be cooled to close to 0°C in 2 to 3 sec. The amount of probe bound to the particles was taken to equal the difference between the amount originally present and that remaining in the supernatant. It is assumed in this method that the room-temperature equilibrium is preserved in the cooled samples. Control experiments showed little or no tendency for dissociation after

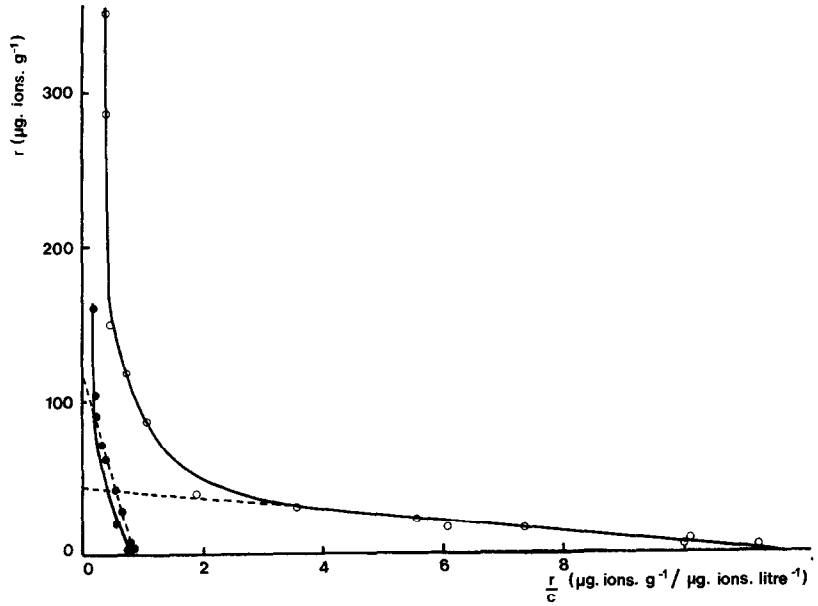

Fig. 1. Scatchard plots of energised o——o and non-energised •——• EDTA particles. Reaction medium was 170 mM sucrose, 10 mM Tris–acetate buffer pH 7.5, 5 mM succinate, 0 (energised) or 1.7 (non-energised) mM KCN, 2 μM rotenone, 1 μg/ml oligomycin and 1.1 mg/ml EDTA particle protein. ANS concentration varied between 2 and 1000 μg. ions litre^{-1}. Values for binding constants, given in table 1, were calculated using the dashed lines.

cooling, but the possibility of some dissociation during the cooling period cannot be completely ruled out.

The binding constants were determined using a version of the equation developed by Scatchard [14] for the analysis of the interaction of small molecules with macromolecules. This states that:

$$r = n - K_d \frac{r}{c} \qquad (1)$$

where r is the number of molecules of probe bound per gram of protein, n is the number of sites per gram of protein, K_d is the apparent dissociation constant and c is the concentration of free probe. For the purpose of this paper r and n are expressed in μg.ions and c in μg.ions litre^{-1}. Assuming this equation to hold (i.e. that the sites can be considered to be homogeneous), a plot of r against r/c should yield a straight line with an intercept on the ordinate of n, and a slope of $-K_d$.

A typical set of results is shown in fig. 1. The Scatchard plots are characteristically bi-phasic, consisting of a linear section of the anticipated form, at high r/c values, and an asymptotic section paralleling the r axis at low r/c values. They are very similar to those obtained by Nordenbrand and Ernster [8] apart from the fact that the asymptotic section is much more clearly marked in the present study. This probably reflects the greater concentration range used in these experiments. If the asymptotic section is ignored, values of n and K_d can be obtained by extrapolation of the linear section of the plot – see fig. 1. This procedure, whilst not necessarily entirely valid, has the advantage of yielding values which can be directly compared to those of earlier studies. The values, which are tabulated in table 1, are similar to those obtained by Ernster and Nordenbrand. The significance of these values will be discussed together with those obtained from the indirect binding studies.

3.2. *Indirect binding studies*

A similar binding analysis was performed by a modified version of the method described by Brocklehurst et al. [3]. This method takes advantage of the

Table 1
Values of binding constants* obtained in this and earlier studies

Direct binding studies

Energised state		Non-energised state			
n (μmoles·g^{-1})	K_d (μM)	n (μmoles·g^{-1})	K_d (μM)	Scatchard characteristics	References
44	4	114	134	Asymptote	Fig. 1
25	3	70	100	Asymptote	[8]

Indirect binding studies

n (μmoles·g^{-1})	K_d (μM)	n (μmoles·g^{-1})	K_d (μM)	Scatchard characteristics	References
22* – 120	2.6* – 120	110	220	No asymptote	Fig. 3b
28* – 70	32* – 180	34* – 74	90** – 260	No asymptote	Fig. 3a
80	20	80	35	No asymptote	[3]
–	–	40 – 45	25	No asymptote	[4]
–	–	200	57	Not drawn	[1]
–	20	–	72	Not drawn	[5]
–	16	–	48	Not drawn	[8]

* Results of present study are expressed in μg.ions and μg.ions litre^{-1} as a divalent salt of ANS has been used.
** Values corresponding to the low concentration range ($\geq$ 40 μM) used in most earlier investigations.

fact that ANS has a very low quantum yield in aqueous solution. This means that all the fluorescence from an ANS-membrane suspension can effectively be attributed to the bound probe. If the fluorescence yield of a given amount of bound ANS is known, it is a simple task to perform the Scatchard analysis by a fluorometric determination of r. The difficulty, however, lies in determining the appropriate value for this fluorescent yield.

The fluorescent yields, f, of a series of membrane suspensions of varying protein concentration, p, were measured at a constant ANS concentration for both energised and non-energised particles. The results, plotted as a double reciprocal graph, are given in fig. 2. The value of the intercept on extrapolation to infinite protein concentration, $f_{(p\to\infty)}$, is normally taken to equal the fluorescent yield of the completely bound probe. A simple linear extrapolation of the plots results in intercepts that differ for energised and non-energised particles. This difference has commonly been interpreted as reflecting an increase in the quantum yield of the bound probe on energisation. However, as pointed out by Harris [6], there is, in fact, no reason to believe that the extrapolation of these plots to infinite protein concentration is necessarily linear; as the probe could well be completely bound even at lower protein concentrations. A close examination of fig. 2 tends to support this view. There is, therefore, considerable doubt as to the correct value for $f_{(p\to\infty)}$ to be used in this type of analysis. For the sake of comparison we have adopted both methods of extrapolation. A simple linear extrapolation, ignoring the higher protein concentrations, of the type used by earlier workers, yields $f_{(p\to\infty)}$ equal to 71 for the energised state and $f_{(p\to\infty)}$ equal to 50 for the non-energised state. Extrapolating the plots as smooth curves, including the higher protein concentrations, yields $f_{(p\to\infty)}$ equal to 36 for both states.

The Scatchard plots calculated from the two sets of values are shown in fig. 3. They are very different. Using the method adopted by the earlier workers, fig. 3a, the plots are linear, or near-linear, with low values of n and high values of K_d. Using the smooth curve extrapolation, fig. 3b, the plot is clearly not linear, the value for n is higher and the values for K_d lower. The strong similarity between the direct binding

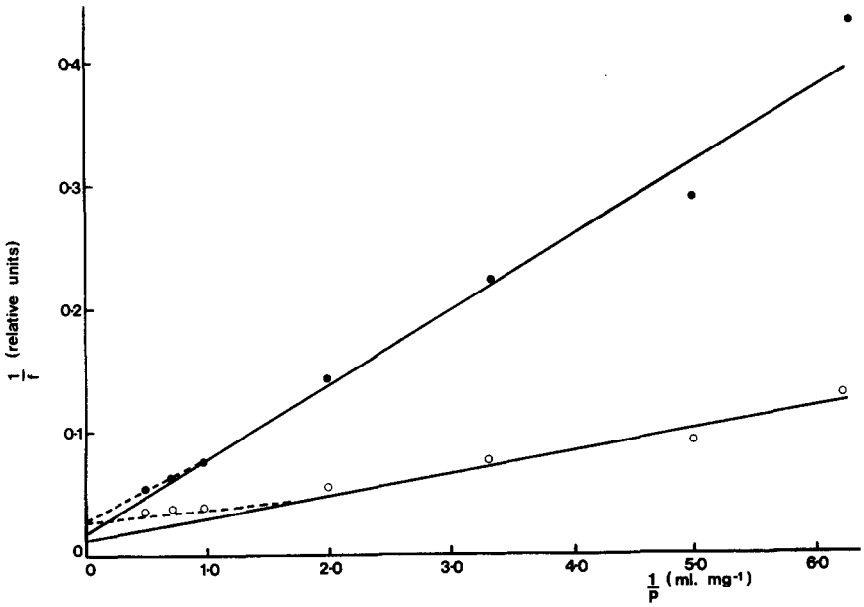

Fig. 2. Double reciprocal plots for the titrations of 16 μg. ions litre^{-1} ANS with energised o——o and non-energised •——• EDTA particles. Reaction medium as fig. 1. EDTA-particle protein varied from 0.16 to 2.0 mg/ml. The straight line extrapolation to the $1/f$ axis is shown by full lines and the smooth curve extrapolation by dashed lines.

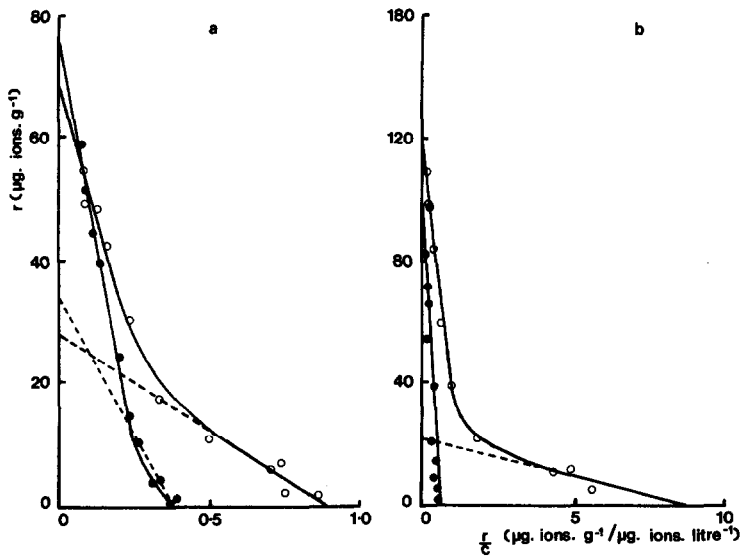

Fig. 3. Scatchard plots calculated from fluorescence measurements for energised o——o and non-energised •——• particles. Reaction medium as fig. 1. EDTA particle protein 1.0 mg/ml. ANS concentration varying from 2 to 800 μg. ions litre^{-1}. (a) Calculated from the straight line extrapolation of the plots in fig. 2. Values of $f_{(p\to\infty)}$ taken as 71 and 50 for energised and non-energised states respectively. (b) Calculated from the smooth curve extrapolation. Value of $f_{(p\to\infty)}$ taken as 36 for both energised and non-energised states. Values for binding constants, given in table 1, were calculated using the dashed lines.

Scatchard plots and the indirect binding plots obtained using the smooth curve extrapolation suggest that this latter method of extrapolation is likely to be the more correct. This is supported by the fact that plots obtained using this method yield values of K_d that correspond closely (in the appropriate concentration range) with those obtained by the direct binding method – see figs. 1 and 3b and table 1.

This tendency for methods involving the extrapolation of fluorescence yield results to give high values of K_d has also been noted by Nordenbrand and Ernster [8]. The marked non-linearity of the Scatchard plots obtained using the smooth curve extrapolation is not surprising. As pointed out by Brocklehurst et al. [3], Scatchard plots of complex systems may contain contributions from many sets of sites and thus should not necessarily be expected to be linear over large concentration ranges. It is possible that the presence of varying concentrations of the fluorescence probe may also influence the shape of the plot. This non-linearity should, however, be distinguished clearly from the biphasic nature of the direct binding Scatchard plots in which a distinct asymptotic section exists.

3.3. *Comparison of direct and indirect binding studies*

A comparison of figs. 1 and 3 indicates two basic differences between the two sets of results, that hold independent of which of the two indirect Scatchard plots is assumed to be the more correct. Firstly, the direct Scatchard plots contain a section asymptotic to the r axis, whilst the indirect plots do not. Secondly, the values of n for the energised and non-energised states are similar, if not identical, for the indirect plots, but differ by a factor of two or more for the direct plots. A comparison of the present results with those obtained by earlier workers, given in table 1, only serves to emphasize these two points.

The immediate questions that arise from these comparisons are: what are the sources of these differences, and what is their significance?

As both the direct and indirect studies are designed to measure identical parameters, it is clear that the theory behind one or both must be at fault. Whilst the obvious shortcomings in the indirect binding theory, outlined above, lead to marked changes in the linearity of the plots, they do not appear to influence the features of the results that we are at present interested in. A far more important shortcoming, in our opinion, lies in the assumption made in the direct binding theory, that all the probe centrifuged down with the EDTA particle pellet is bound to the membrane.

The parameter r of the Scatchard equation is normally calculated directly from the amount of probe in the pellet. This does not take account of the possibility that the probe in the pellet may be either in a free or a bound state. No direct statement as to whether the mitochondrial membrane is permeable to ANS appears to exist in the literature. Some workers have tacitly assumed it to be impermeable, others have assumed it to be permeable. We measured the fluorescence of ANS in a particle suspension before and after sonication. Sonication would be expected to disrupt the particles allowing access of ANS to new sites in their interior. If the probe is permeable, these sites should already be in equilibrium with the probe. If the probe is impermeable, the exposure of new sites on sonication should increase the total binding and this should be reflected in an increased fluorescence yield. Despite the fact that there was free ANS present in the suspension, no increase in fluorescence was observed on disrupting the particles. From this, we conclude that all possible binding sites were already in equilibrium with the probe, i.e. the membrane is freely permeable.

The value of r used in the original analysis, therefore, consists of two terms:

$$r = r_b + r_f \tag{2}$$

where r_b and r_f refer to the bound probe and the free probe contained within the particle respectively. The value of r_f will equal the product of the internal concentration of free probe, c_{in}, and the net internal volume of the particles, v_{in}. As in eq. (1) it is assumed that the particles are effectively homogeneous. Substituting for r_f in eq. (2) and dividing by the external concentration of free probe (originally defined as c but now conveniently redefined as c_{out}) we obtain:

$$\frac{r}{c_{out}} = \frac{r_b}{c_{out}} + \frac{c_{in}v_{in}}{c_{out}} \tag{3}$$

If we assume the internal and external concentrations of free probe are in equilibrium and that this equilibrium is determined by the degree of energisation of the particles, we can write:

$$K = \frac{c_{in}}{c_{out}} \quad (4)$$

where K is a constant. Combinations of equations (3) and (4) then yield:

$$\frac{r}{c_{out}} = \frac{r_b}{c_{out}} + Kv_{in} \quad (5)$$

This equation indicates that the quantity r/c measured in the direct binding studies in fact consists of two terms. The first of these is a variable term r_b/c_{out} (i.e. the parameter of interest), and the second term Kv_{in}, is a constant for a given degree of energisation. From this analysis the explanation of the bi-phasic nature of the direct binding Scatchard plots is clear. The asymptotic section corresponds to Kv_{in} and the linear section corresponds mainly to r_b/c_{out}. The absence of the asymptotic section in the indirect plot reflects the fact that the measurements of r in these studies relate directly to r_b, with no contribution from r_f.

The difference in the intercept values between the energised and non-energised plots in the direct binding studies can readily be explained by the distortion of the binding curve by the asymptotic sections. This is particularly marked for the non-energised plot and results in a gross over-estimation of the value of n in this case. This distortion does not appear in the indirect studies. The general conclusion from these latter studies that n does not change on energisation, therefore, seems to be valid , even if its exact value still remains in some doubt.

Three basic points emerge from the above analysis. Firstly, that if the allowance is made for free probe contained within the particles and the appropriate extrapolation methods are used, both the direct and the indirect methods of determining Scatchard plots give essentially similar results. Secondly, that the number of potential binding sites is independent of the state of energisation. This precludes interpretations of the energisation phenomenon, of the type offered by Azzi [2] and by Nordenbrand and Ernster [8], involving the formation of new binding sites. Thirdly, that the ratio of c_{in} to c_{out}, and hence the value of K_d appears to be controlled by an equilibrium constant, the value of which is determined by the degree of energisation of the system.

We therefore suggest the following interpretation, based on the model formulated by Jasaitis et al. [7], for the energisation process. Coupled electron transport, initiated by the addition of succinate, leads to the accumulation of protons by the particles. This results in the interior of the particles becoming positively charged with respect to the external solution. ANS being negatively charged will tend to accumulate within the particles in order to neutralise this excess charge. The steady state concentration of ANS within the particles will thus increase. The size of this increase will depend on the rate of accumulation of protons and the magnitude of the resulting membrane potential.

The absolute amounts of ANS accumulated by the particles will be small. However, as pointed out by Jasaitis et al. [7], the net internal volume of the particles is small with respect to the total volume of the suspension and thus the relative change in ANS concentration will be much greater inside than outside the particles. It is this relatively large change in the internal concentration of free ANS leading to a displacement of the equilibrium between free and bound probe inside the particles that results in the observed increase in binding that accompanies energisation.

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