Biochimica et Biophysica Acta, 974 (1989) 156–162
Elsevier

BBA 42973

Azide as a probe of co-operative interactions in the mitochondrial F_1-ATPase

David A. Harris

Department of Biochemistry, University of Oxford, Oxford (U.K.)

(Received 25 October 1988)

Key words: ATPase, F_1-; Co-operativity; Azide inhibition; Unisite catalysis; Inhibitor protein, IF_1

(1) The hydrolytic activity of the isolated mitochondrial ATPase (F_1) is strongly inhibited by azide. However, at very low ATP concentrations (1 μM or less), no inhibition by azide is observed. (2) The azide-insensitive ATPase activity represents a high-affinity, low-capacity mode of turnover of F_1. This is identified with the low K_m, low V_{max} component seen in steady-state kinetic studies in the absence of azide. (3) The azide-insensitive ATPase activity shows simple Michaelis-Menten kinetics, with $K_m = 3.2$ μM, and $V_{max} = 1.1$ μmol/min per mg (6 s^{-1}). It is unaffected by anions such as sulphite, or by increasing pH in the range 7 to 8, both of which stimulate the maximal activity of F_1. (4) Both the azide-insensitive and azide-sensitive components of F_1-ATPase activity are equally inhibited by labelling the enzyme with 7-chloro-4-nitrobenzofurazan, by binding the natural inhibitor protein, or by cold denaturation of the enzyme. (5) It is concluded that azide-insensitive ATP hydrolysis represents catalysis by F_1 involving a single catalytic site, and that azide acts by abolishing intersubunit cooperativity between the three catalytic sites of F_1. Azide-sensitivity is thus a useful probe for events which affect the active site of F_1 directly.

Introduction

The mitochondrial ATP synthase (F_1-ATPase), and its homologues from other sources, bears three nucleotide binding sites that participate in catalysis [1,2]. Each binding site occupies an equivalent position on one of the three β subunits of this enzyme [3,4]. However, affinity for ATP of each binding site decreases as a previous site is filled, from $K_d = 10^{-12}$ M for the first binding site to $K_d \geq 1$ μM for the third. It is uncertain whether this change in affinity reflects asymmetry induced by ATP binding to one of three equivalent subunits (classical 'negative co-operativity') or, alternatively, an asymmetry pre-existing in the enzyme in the absence of added nucleotide (for a review, see Ref. 5).

Steady-state kinetics studies on the F_1-ATPase by Gresser et al. [6] revealed turnover at two classes of active site, one with $K_m^{MgATP} \approx 1$ μM and another, with V_{max} some 50-fold larger, with $K_m = 250$ μM. The increased V_{max} as ATP concentrations are raised is diagnostic of positive co-operativity between catalytic sites. Such co-operativity was also deduced by studying the nucleotide dependence of P_i–H_2O exchange catalysed by isolated F_1 [7]. A combination of negative cooperativity in binding, and positive cooperativity in catalysis, is characteristic of enzymes operating by an alternating site mechanism; how far this model applies to F_1 depends on an assessment of the significance of pre-existing asymmetry in the function of the enzyme.

It should, of course, be possible to correlate binding measurements with kinetic data for this enzyme. For this reason, it is disconcerting that the number of measured (exchangeable) binding sites for ATP is three [1,2,9], while the number of K_m values measured in [6] is only two. Furthermore, there is a large difference in magnitude between the lowest K_d value at 10^{-12} M [10], and the lowest K_m at 10^{-6} M [6,11,12]. One possibility, put forward by Cross et al. [10] and favoured by a number of workers [11,13,14] is that when only the first binding site is occupied by ATP, catalysis (termed 'unisite') is so slow as to be negligible in steady-state analysis. In this view, the low V_{max} value observed is the result of turnover of an enzyme with two of its three catalytic sites filled ('bisite catalysis').

Gresser et al. [6] proposed an alternative view, in which the lowest K_m represents 'unisite catalysis', viz. turnover with only one of the three catalytic sites operative. This is supported in some measure by the ob-

Abbreviations: F_1, soluble mitochondrial ATPase from bovine heart; IF_1, naturally occurring inhibitor protein of F_1; Nbf-Cl, 7-chloro-4-nitrobenzofurazan; Mops, 4-morpholinepropanesulphonic acid.

Correspondence: D.A. Harris, Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, U.K.

servation of *two* higher K_m values in more recent studies on ATP hydrolysis by F_1 [8,11], but this interpretation is still equivocal (see Ref. 11). Problems arise partly because of the discrepancy between K_d and K_m, and partly because the properties of the high-affinity, low-capacity turnover could not be studied in isolation from co-operative effects within the enzyme.

A convenient way to study unisite catalysis would be to use an enzyme preparation in which co-operative interactions have been abolished. This has been achieved in the *E. coli* enzyme, where a non-cooperative mutant (*uncA401*) is available [15], and in the enzyme from *Rhodospirillum rubrum*, where a catalytically active β subunit can be isolated, lacking the influence of the other subunits [16]. In the work below, we show that, in the mitochondrial F_1-ATPase, co-operative interactions can be abolished by the non-competitive inhibitor, azide. This is exploited to investigate the effects of co-operative interactions in catalysis, and to allocate the mode of turnover with $K_m \approx 1$ μM to unisite catalysis. It is also used to show that both the covalent inhibitor, Nbf-Cl, and the non-covalent natural inhibitor protein, IF_1, inhibit catalysis by F_1, directly, and not simply by preventing intersubunit cooperation.

Materials and Methods

F_1 and IF_1 were prepared from ox heart mitochondria as previously [36]. F_1 was freed from medium nucleotides by centrifugation, twice, through sequential Sephadex G-50 columns, and ATP hydrolysis measured, at 37 °C, in 60 mM sucrose/50 mM Tris/50 mM KCl/2 mM $MgCl_2$/1 mM EDTA (pH 8.0) (HCl), using [γ-^{32}P]ATP, essentially as described previously [16]. When ATP concentrations exceeded 500 μM, phospho*enol*-pyruvate (1 mM) and pyruvate kinase (1 U/ml) were additionally present in the assay medium to remove ADP. Reaction times were typically 1 min, and less than 5% of added ATP was hydrolysed.

Under these conditions, the rate of product release was constant for up to 2 min, whether or not azide was present. This finding contrasts with the observation of Daggett et al. [17] that, in the presence of azide, ATP hydrolysis falls off in time, i.e., azide inhibition develops over a 1–2 min period of turnover. The reason for this difference seems to lie in the assay temperature; at 30 °C, we observe a significant lag in azide inhibition (only 20% of final level in the first 30 s), in agreement with Ref. 17, while at 37 °C, used below, inhibition is virtually complete within 30 s (data not shown).

Protein concentrations were determined with a proprietary reagent from Bio-Rad laboratories, based on the method of Bradford [23]. Nucleotides and enzymes were obtained from Boehringer, and Nbf-Cl from Aldrich.

Results

Effects of azide on the hydrolytic activity of F_1

The effect of azide on ATP hydrolysis by mitochondrial F_1 was measured at 2 mM ATP (a high concentration of ATP, when all three catalytic sites are operative) and at 0.86 μM ATP. Fig. 1 shows, in agreement with previous work [17,18], that hydrolysis of millimolar concentrations of ATP is strongly inhibited by azide ($c_{1/2} < 0.1$ mM). However, hydrolysis of 0.86 μM ATP was hardly affected by azide (rate with 1 mM azide = 93 ± 4% of the rate without azide, $n = 6$). Hydrolysis of ATP at both concentrations was linear over the course of the experiment, whether or not azide was present (data not shown). Note that pyruvate kinase and phosphoenolpyruvate were included in the assay at 2 mM ATP, to remove ADP formed.

A lack of azide inhibition at low ATP concentrations was also observed, with *E. coli* F_1, by Noumi et al. [19], and the mitochondrial ATPase thus appears similar. However, in Ref. 19, hydrolysis was measured under conditions where free ATP was negligible (single turnover conditions; $e_0 = s = 0.5$ μM). Here we measure hydrolysis in the steady state (at 0.15 nM F_1) and hence can follow the dependence of azide inhibition on free ATP concentration. Fig. 2 shows that, as ATP levels fall, inhibition by azide becomes progressively less severe, falling from around 90% at 2 mM ATP to essentially zero as ATP falls below 0.5 μM. Since, as ATP concentrations approach zero, the activity of F_1 tends towards a unisite mode, it is concluded that azide inhibits multisite but not unisite catalysis on the

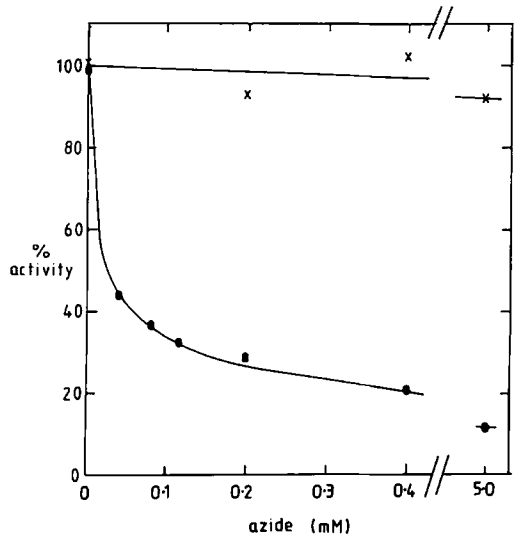

Fig. 1. Effect of azide on hydrolysis at high and low ATP concentrations. 0.4 ml reaction medium (see Materials and Methods) contained either 2 mM MgATP (300 dpm/nmol) or 0.86 μM MgATP ($700 \cdot 10^3$ dpm/nmol) and the indicated amount of sodium azide. The reaction was initiated, at 37 °C, by the addition of F_1 (1 μg or 0.05 μg respectively) and stopped, after 1 min, by the addition of trichloroacetic acid to 4% (w/v). ●, 2 mM MgATP; ×, 0.86 μM MgATP.

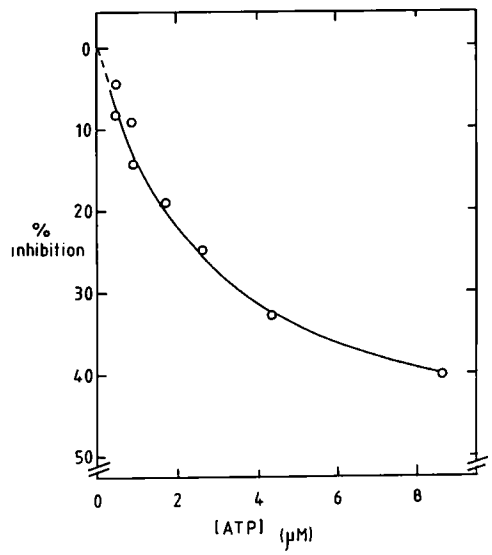

Fig. 2. Variation of inhibition by azide with ATP concentration. ATPase activity was followed as in Fig. 1, except that the azide concentration was fixed at 1 mM, and ATP concentrations in the reaction medium varied as indicated.

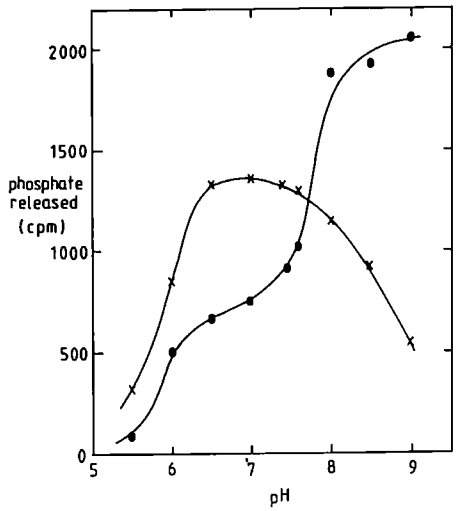

Fig. 4. pH dependence of low- and high-capacity ATPase activity. The low-capacity ATPase was measured at 0.86 μM MgATP in the presence of 1 mM azide, and the high-capacity ATPase at 2 mM MgATP, as in Fig. 1. HCl was omitted from the reaction medium, and the pH varied by the addition of varying amounts of Mops. ●, 2 mM MgATP; ×, 0.86 μM MgATP.

mitochondrial F_1-ATPase. This is in agreement with conclusions drawn from the *E. coli* and *R. rubrum* enzymes [15,16,19].

An obvious mechanism for this differential effect is that azide inhibits intersubunit cooperativity in F_1. This postulate is confirmed in the experiment of Fig. 3, in which the effect of ITP on the hydrolysis of low concentrations of ATP is tested. ITP is chosen as an effector because it is readily hydrolysed by F_1, but it cannot bind to the 'regulatory' nucleotide binding sites of this enzyme [9,20]. Thus, its effects must reflect events at the catalytic sites of F_1.

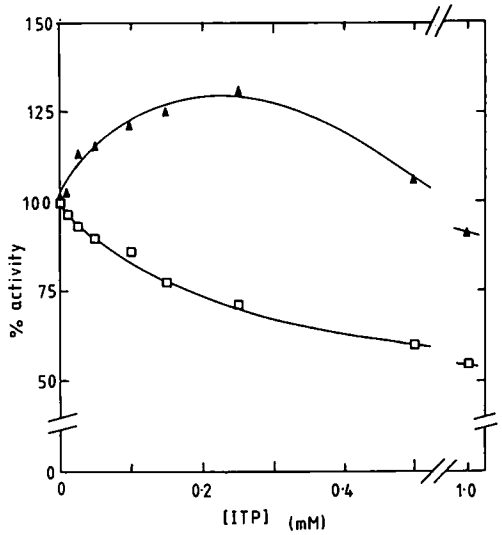

Fig. 3. Effect of ITP on hydrolysis of 0.86 μM ATP. ATPase activity was followed as in Fig. 1, except that the MgATP concentration was maintained at 0.86 μM, and MgITP varied as indicated. ▲, azide omitted; □, 1 mM azide present.

Cooperativity in F_1 is demonstrated in the upper curve of Fig. 3. ITP, despite its ability to compete with ATP for the active site of F_1, stimulates hydrolysis of a small concentration of radiolabelled ATP, as previously shown [21,22]. As can be seen from the lower curve of Fig. 3, azide abolishes this cooperativity and ITP now exhibits typical inhibitory properties. These results are similar to those observed when comparing ATP hydrolysis by *R. rubrum* F_1 (cooperative) and by its β subunit (not cooperative) [16]. It is concluded that azide exerts its inhibitory effect by blocking intersubunit cooperativity in F_1, and that this effect is not observed at very low ATP concentrations where further sites involved in cooperation remain empty.

Properties of the azide-insensitive ATPase of F_1

The azide-insensitive ATPase activity of F_1 differs in several properties from its multisite activity. One manifestation of this difference is shown in Fig. 4, which shows the pH dependence of both activities. As has been well documented [18], the multisite activity of F_1 increases markedly over the pH range 7–8, maximal activity being observed only above pH 8. However, Fig. 4 shows that the azide-insensitive ATPase activity is largely unaffected by pH in the range 7–8, and indeed falls above pH 8. This may indicate that the group ionising with $pK_a \approx 7.5$, which dominates the pH dependence of F_1 at physiological ATP concentrations, is not an (acid-base) catalytic group but, rather, a group involved in intersubunit cooperativity. Both azide sensitive and insensitive activities show another ionising group ($pK_a < 6.5$) affecting turnover rate (Fig. 4), but whether this is a group on the enzyme or the substrate, MgATP, is not clear from these data.

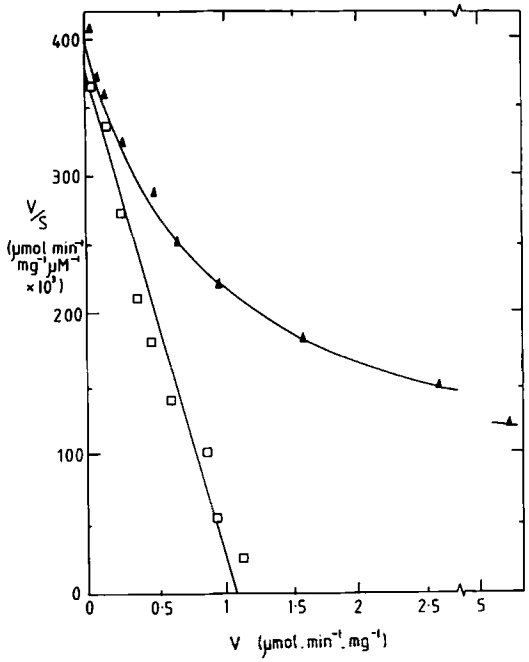

Fig. 5. Dependence of turnover rate on substrate concentration – effect of azide. ATP hydrolysis, at ATP concentrations in the range 0.1–43 μM, was measured in the presence or absence of 1 mM azide, as in Fig. 2. To correct for a small fraction (12% – see Fig. 1) of high capacity turnover at 1 mM azide, the true azide-insensitive rate (v) was calculated using the formula

$$v = v_1 - \frac{0.12}{0.88}(v_2 - v_1)$$

where v_1 is the rate observed in the presence of 1 mM azide, and v_2 the rate observed in its absence. The correction was less than 10% below 10 μM MgATP. ▲, azide absent; □, 1 mM azide present.

From Fig. 2 above, it is clear that, at low ATP concentrations, the azide-insensitive ATPase activity of F_1 predominates, while at high ATP concentrations, the azide-sensitive activity is by far the higher. This implies that the azide-insensitive activity has the higher affinity for ATP. This is confirmed in Fig. 5, in which the kinetic parameters of this ATPase are measured directly. As can be seen, in the absence of azide, an Eadie-Hofstee plot of the kinetic data (between 0.1–43 μM MgATP) is concave upwards – indicating the operation of more than one ATP binding site, with progressively weaker binding. However, in the presence of azide (and applying a small correction to allow for incomplete inhibition by 1 mM azide), the curvature disappears and only a single class of ATP binding site appears to operate.

Towards low substrate concentrations (low v), the two curves in Fig. 5 can be seen to approach other, i.e., the behaviour of the enzyme with and without azide becomes similar. This confirms that the azide-insensitive ATPase activity is a normal mode of functioning of this enzyme and not an abnormal mode induced by the presence of azide itself. The K_m and V_{max} for this mode of functioning of the enzyme are respectively 3.2 μM and 1.1 μmol/min per mg (6 s^{-1}), from Fig. 5. This compares with the maximum rate of this enzyme preparation of 45 μmol/min per mg (270 s^{-1}), with K_m = 300 μM (measured over the region 80–5000 μM) (data not shown). Thus the azide-insensitive ATPase is a high-affinity, low-capacity mode of turnover by this enzyme.

The values of K_m and V_{max} obtained from Fig. 5 are very close to the values observed, from curve-fitting multisite kinetic data, by Gresser et al. [6] who measure $K_m = 1.7$ μM, and $V_{max} = 2.2$ μmol/min per mg for their high-affinity catalytic activity. However, they are very different from any of the values calculated for these kinetic parameters by Cross et al. [10]. This is discussed further below.

Another difference is observed in the sensitivities of these modes of turnover to activating anions. Hydrolysis of 2 mM ATP by F_1 is stimulated 70% by the addition of 5 mM sodium sulphite to our assay medium (see Materials and Methods). In contrast, hydrolysis of 0.86 μM ATP is unaffected by sulphite, whether or not azide is present (data not shown). The insensitivity of hydrolysis of submicromolar concentrations of ATP to bicarbonate, another stimulatory anion, has been shown by Kasho and Boyer [22]. Thus the high-affinity, low-capacity ATPase activity of mitochondrial F_1 is insensitive to both inhibitory (azide) and stimulatory (sulphite, bicarbonate) anions.

Mode of action of 'irreversible' inhibitors of F_1

From above, it seems that azide inhibits F_1 turnover solely by blocking intersubunit cooperativity; it does not interfere directly with events at the active site of F_1. The covalent inhibitor, Nbf-Cl, which binds at one of the (three) presumed active sites of F_1 [24,25], shows contrasting behaviour. Fig. 6 shows that, as Nbf-Cl labels F_1, both the low- and high-capacity ATPase activities fall in parallel. This parallel is maintained when the Nbf label is removed by dithiothreitol, and activity is regained (Fig. 6). These effects of Nbf-Cl can be explained solely by its inhibiting at an active site of F_1; this will, of itself, interfere with co-operation between all three active sites in the labelled enzyme. This finding is consistent with the inhibitory effect of Nbf-labelling on ATP binding to F_1, as observed by Bullough et al. [26].

Such behaviour is not unexpected from a covalent inhibitor, like Nbf-Cl, which is believed to bind to an active site on F_1. Inhibition of F_1 by its natural inhibitor protein IF_1 (believed to bind at a site distant from catalytic residues [27]), or by cold treatment (which leads to enzyme dissociation [28]) are less predictable in their outcome. Nonetheless, Table I shows that either of these treatments leads to parallel losses in multisite (high capacity) and azide-insensitive (low capacity) activities of F_1. Loss of 50% multisite ATPase activity, by either treatment, is accompanied by a similar loss in the

TABLE I

Effects of IF_1 and cold denaturation on the low-capacity ATPase activity of F_1

F_1 was freed from salt and nucleotides as described in Materials and Methods. F_1 (1 mg/ml) was combined with IF_1 (1:10 molar ratio) in the presence of MgATP at pH 6.7, as described in [27], and nucleotides removed by centrifugation through a further Sephadex G-50 column. Alternatively, F_1 was partially cold-denatured by incubation at a concentration of 0.4 mg/ml, at 0 °C for 90 min. ATPase activity, in the presence of sodium azide (1 mM) and/or ITP (200 μM) was measured at 0.86 μM ATP (low ATP) or 2 mM ATP (high ATP) as in Fig. 1. (Values given ± the range of five readings where indicated.)

Pretreatment	Relative ATPase activity					
	low ATP				high ATP	
	no addition	+ azide	+ ITP	+ azide + ITP	no addition	+ azide
		% rate with no addition	% rate with no addition	% rate with no addition		% rate with no addition
None	100	93 ± 7	100 ± 9	70 ± 8	100	11 ± 1
+ IF_1	52	92	115	67.5	56	14
Cold	36	89	114	64	34	11

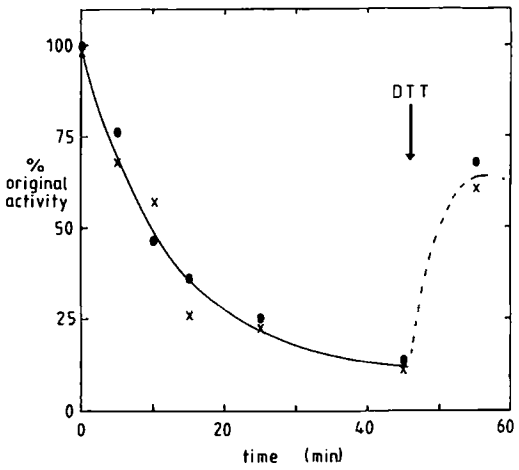

Fig. 6. Effect of Nbf-labelling of F_1 on high- and low-capacity ATPase. F_1 (1 mg/ml) was labelled with Nbf-Cl (50 μM) as described in Ref. 25. At intervals, aliquots were removed and the reaction quenched by dilution into 100 mM Tris/2 mM EDTA (pH 8.0) (HCl), followed by column centrifugation (see Materials and Methods). After the measured activity had declined to less than 20%, dithiothreitol (DTT) (1.5 mM) was added to the quenched sample, and the mixture was incubated at 25 °C for 15 min. ATPase activity was measured at 0.86 μM (×) or 2 mM MgATP (●) as in Fig. 4.

azide-insensitive activity (left-hand columns). Treatment of F_1 by IF_1 (to 50% inhibition), followed by cold inactivation (to less than 20% activity) shows that the effects are additive (data not shown). An inhibitory effect of IF, on hydrolysis by F_1 of very low ATP concentrations has recently been reported by Kalashnikova et al. [29].

The remaining columns of this table show that the residual low capacity ATPase, after either of these treatments, is unaltered – a small stimulation by ITP, and inhibition by azide in the presence of ITP, is observed just as for untreated F_1 (see Fig. 3). This is consistent with the view that all the ATPase activity observed comes from unmodified F_1 molecules, and thus that the modified F_1 has negligible amounts (less than 10%) of its original low-capacity ATPase activity. It can be concluded that both IF_1 binding and cold treatment exert their effects at the catalytic sites of F_1, and not simply by preventing intersubunit cooperation. This is discussed further below.

Discussion

Catalysis by F_1 – how many sites?

It is shown above that azide blocks intersubunit cooperativity in F_1 (Figs. 3, 5), revealing a mode of catalytic turnover with $K_m = 3$ μM and $V_{max} = 1$ μmol/min per mg (6 s^{-1}) (Fig. 5). These values agree well with those measured by a number of groups [6,8,11] for the high-affinity ATP hydrolytic site, by fitting steady-state kinetic data with a multiple-site model. Thus, azide allows the direct demonstration (and investigation) of the properties of F_1 in a low-capacity mode of turnover, previously only inferred from simulations. In the absence of azide, as ATP levels are raised, F_1 turnover is by a higher-capacity mode (involving intersubunit cooperation) with $K_m = 300$ μM and $V_{max} = 50$ μmol/min per mg (300 s^{-1}) [12].

As noted above, F_1 has three catalytic binding sites for nucleotides, one on each of its three β subunits [1–3,9]. The simplest interpretation of our kinetic data, thus, might be that only two of these binding sites are involved in ATP hydrolysis, the remaining site having some other function (e.g., ATP synthesis). This hypothesis has been put forward by a number of workers on the basis of labelling and reconstitution studies [13,14,26].

However, the differential anion dependence of the low- and high-capacity ATPase activities cannot be fitted to a two site model for turnover. As demonstrated above, sulphite (and bicarbonate [22]) stimulate hydrolysis by F_1 in its high-capacity, but not in its low-capacity, mode of turnover. This means that the Eadie-Hofstee plot for ATP hydrolysis over the range shown in Fig. 5 (0.1–43 μM) will increase in curvature in the presence of activating anions, as the kinetic parameters

of the two modes of turnover become more divergent in the presence of anions. However, as shown initially by Ebel and Lardy [30] (and subsequently by many other workers – see, for example, Ref. 22), anions abolish the curvature of such kinetic plots over the range 50–2000 μM ATP. This can occur only if the first catalytic site (highest affinity) is anion-insensitive and two others (which normally give rise to apparent negative cooperativity in the upper concentration range) are anion-sensitive. This interpretation is supported by the kinetic studies of Wong et al. [11], and Roveri and Calcaterra [8], who demonstrate three K_m values for hydrolysis; in the presence of stimulatory anions, they find that the lowest K_m value is unaltered, but the two higher values approach one another, and may become indistinguishable [8].

This last point further implies that azide- and sulphite-insensitive ATP hydrolysis represents unisite turnover of this enzyme. This is consistent with the kinetic analysis of Gresser et al. [6], who showed that the ATP dependence of $H_2^{18}O \leftrightarrow P_i$ exchange was incompatible with a two-site model, and also postulated a three-site model with a low-capacity, high-affinity site (turnover $\approx 10\ s^{-1}$) representing the unisite (uncooperative) mode of turnover. We conclude, therefore, that all three catalytic sites of F_1 are involved in ATP hydrolysis in the physiological range, and that the properties of the unisite mode of turnover are those of the azide-insensitive ATPase activity measured above.

In contrast, Cross et al. [10] deduce a turnover number some 10^5-times lower, and a K_m some 10^6-times lower, for unisite catalysis. To follow the latter analysis, azide-insensitive hydrolysis would represent bisite catalysis by F_1 – catalysis in which two catalytic sites cooperate and only the third is inoperative.

This latter model is unattractive for at least two reasons. First, azide would have to distinguish between two subunits acting cooperatively (not inhibited) and three acting cooperatively (inhibited), which seems unlikely. Secondly, the values of K_m and V_{max} proposed for the second site by Cross et al. [10] are 30 μM and 300 s^{-1}, respectively, both an order of magnitude higher than the values measured here for azide-insensitive ATPase. So, while this model cannot be totally ruled out, we conclude from the above studies that it is unisite catalysis which is insensitive to azide.

Why, then do the values of $K_m = 10^{-12}$ M and $V_{max} = 10^{-4}\ s^{-1}$ for unisite hydrolysis emerge from the work of Cross et al. [10]? This is probably because neither of these parameters is, in fact, measured during steady-state hydrolysis. In Ref. 10, K_m is taken as close to K_d for binding of ATP to the first site of F_1. However, in the reaction

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + \text{products}$$

if $k_2 \ll k_3$ (as here), $K_m = k_3/k_1$ – and taking a steady-state turnover rate of $k_3 = 10\ s^{-1}$ (above) and $k_1 = 10^7\ M^{-1}\ s^{-1}$ [6,12] – a K_m of around 10^{-6} M should be observed, as is seen above. Secondly, V_{max} in Ref. 10, is estimated from the off-rate of ADP, taken to be linear, at $e_0 > s$ [31]. Since, however, no trap for free ADP was available in these experiments, an exponential relaxation towards a new equilibrium will be observed – and attempt to measure the ADP off-rate from the tail-end of this relaxation (as is attempted in Fig. 5 of Ref. 31) will lead to severe underestimation.

It is thus concluded that, contrary to initial expectations, the data relating to 'unisite catalysis' (strictly 'single turnover experiments') in Ref. 31 are consistent with the parameters for the low-capacity ATPase activity measured above (and in Ref. 6, 8, 11). Further, this means that the ADP off-rate, rather than being 5 orders of magnitude lower than the chemical turnover step of F_1 (as in Ref. 10) is in fact of the same order of magnitude. This is more in keeping with the properties of enzymes in general (cf. Ref. 32), and, in particular, is true of the two 'uncooperative' preparations known – the uncA401 mutant of *E. coli* [15] and the β subunit of the *R. rubrum* ATPase [16] – suggesting that a similarity of these two rates may indeed be expected under 'unisite' conditions.

To summarise, it is concluded that the low-capacity, azide-insensitive activity of mitochondrial F_1 represents catalysis with only one of the three sites of F_1 operating. The discrepancy between the kinetic parameters measured for this activity, and the parameters deduced by Cross et al. [10], lie largely in the different modes of measurement – above (and in Refs. 6, 8, 11) using steady-state conditions ($e_0 \ll s$), and in [10,31], using single turnover conditions ($e_0 > s$). Activity in the unisite mode is responsible for the $H_2^{18}O \leftrightarrow P_i$ exchange activity observed during hydrolysis of ATP by F_1.

Azide-insensitive ATPase is also insensitive to anions such as sulphite and bicarbonate, which stimulate multisite catalysis by F_1, and to pH in the range 7–8 (Fig. 5). These differential effects suggest that the rate-limiting steps in the two modes of turnover differ (see Refs. 12, 22). It is likely from measured rate constants, however, that ADP release actually limits turnover in both modes [6,10,12,22]. We are thus left with the conclusion that ADP release in the multisite mode must be preceded by the true rate-limiting step, a conformational change, which is affected by all these agents, and which is absent during unisite turnover (without intersubunit cooperativity). Deprotonation of a non-active site residue ($pK_a \approx 7.5$) facilities this change.

Inhibitor sensitivities of unisite catalysis

Using azide-insensitive ATP hydrolysis as a probe of unisite activity, it is concluded that Nbf-labelling, cold denaturation of F_1, and binding the natural inhibitor

protein, IF_1, all inhibit unisite activity and thus, in some way, affect the active site of this enzyme (Fig. 5; Table I).

Nbf-Cl is a covalent label, which binds to Tyr-311 on the β subunit of F_1 [24,25]. This residue probably lies within the active site of F_1 – as shown by competition studies with P_i [33], and sequence comparisons [3,4]. The action of Nbf-Cl on unisite catalysis is thus consistent with a direct action at the active site.

IF_1 binds to the catalytic subunit of F_1 [34,35] and is inhibitory at a 1 : 1 molar ratio [36]. Its only known point of contact with the β subunit is close to its C-terminus, distant from the nucleotide binding domain [27]. The data given in Table I, however, and similar results reported by Kalashnikova et al. [29], show that IF, inhibits unisite catalysis and point to a direct effect of IF_1 on the active site of F_1. This could be explained by an additional point of contact of IF_1 with the active site, not detected in our previous crosslinking experiments [27], or by a conformational change induced by IF_1 binding. Most likely, however, IF_1 binding (which requires MgATP) is accompanied by trapping one or more nucleotides at the active site(s) of F_1 [37], thus blocking the active site indirectly via the trapped nucleotide molecule.

This idea is consistent with studies on the inhibitory action of the ϵ subunit of *E. coli* and chloroplast F_1. This subunit acts similarly to IF_1, inhibiting these non-mitochondrial ATPases at a 1 : 1 molar ratio [38,39]. Its binding (in the *E. coli* system) involves the C terminal region of the β subunit [40]. In contrast to IF_1, the ϵ subunit of *E. coli* [41] or chloroplasts (Andralojc and Harris, unpublished data) does not inhibit unisite activity, but only the multisite mode. Continuing the above argument, this may be related to the absence of a requirement for ATP in binding the ϵ subunit in these systems.

The effect of cold denaturation on the unisite mode of F_1 activity was unexpected, since the β subunit of F_1, while it changes its association with the other subunits, should remain intact during cold treatment. However, irreversible changes do occur on cold denaturation, and dissociation itself decreases the affinity of the enzyme for nucleotides [28], so as yet undefined active-site changes may well occur.

Acknowledgments

I thank J. Andralojc and A.M. Das for helpful discussions, and the British Heart Foundation for financial support.

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