Effects of Dimethyl Sulfoxide on Catalysis in *Escherichia coli* F_1-ATPase†

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ABSTRACT: (1) Dimethyl sulfoxide (DMSO) markedly inhibited the V_{max} of multisite ATPase activity in *Escherichia coli* F_1-ATPase at concentrations >30% (v/v). V_{max}/K_M was reduced by 2 orders of magnitude in 40% (v/v) DMSO at pH 7.5, primarily due to reduction of V_{max}. The inhibition was rapidly reversed on dilution into aqueous buffer. (2) K_d^{ATP} at the first, high-affinity catalytic site was increased 1500-fold from 2.3×10^{-10} to 3.4×10^{-7} M in 40% DMSO at pH 7.5, whereas K_d^{ADP} was increased 3.2-fold from 8.8 to 28 μM. This suggests that the high-affinity catalytic site presents a hydrophobic environment for ATP binding in native enzyme, that there is a significant difference between the conformation for ADP binding as opposed to ATP binding, and that the ADP-binding conformation is more hydrophilic. (3) Rate constants for hydrolysis and resynthesis of bound ATP in unisite catalysis were slowed ~10-fold by 40% DMSO; however, the equilibrium between bound P_i/bound ATP was little changed. The reduction in catalysis rates may well be related to the large increase in K_d^{ATP} (less constrained site). (4) Significant P_i binding to *E. coli* F_1 could not be detected either in 40% DMSO or in aqueous buffer using a centrifuge column procedure. (5) We infer, on the basis of the measured constants K_a^{ATP}, K_2 (hydrolysis/resynthesis of ATP), k_{+3} (P_i release), and K_d^{ADP} and from estimates of k_{-3} (P_i binding) that ΔG for ATP hydrolysis in 40% DMSO-containing pH 7.5 buffer is between −9.2 and −16.8 kJ/mol.

Membrane-bound F_1F_o-ATP synthase enzymes catalyze ATP synthesis by oxidative or photophosphorylation. In vivo, proton gradient energy provides the driving force for ATP synthesis. The F_1 sector, carrying the catalytic nucleotide-binding sites, catalyzes rapid ATPase when released in soluble form from the membrane (Boyer, 1989; Senior, 1990). Turnover of ATP synthesis has not been seen with soluble F_1, but there are several lines of evidence suggesting that, at a single high-affinity catalytic site, the ATPase reaction on soluble F_1 is reversible (Hutton & Boyer, 1979; Choate et al., 1979; Bossard et al., 1980; Feldman & Sigman, 1982; Grubmeyer et al., 1982; Duncan & Senior, 1985; Wood et al., 1987). Sakamoto and colleagues (Sakamoto & Tonomura, 1983; Sakamoto, 1984a,b) demonstrated that net ATP synthesis could occur on soluble mitochondrial F_1 in the presence of dimethyl sulfoxide (DMSO).[1] The ATP appeared to form at a single catalytic site in amounts substoichiometric with F_1 and to remain firmly bound to the enzyme. Subsequently, this phenomenon was confirmed by Gomez-Puyou et al. (1986) and Kandpal et al. (1987) and demonstrated also with soluble F_1 from the thermophilic bacterium PS3 (Yoshida, 1983).

Rationales for the promotion of ATP synthesis in soluble F_1 by DMSO have been discussed by Kandpal et al. (1987) and deMeis (1987, 1989). While ADP binding to catalytic sites of soluble F_1 appeared little-affected (Sakamoto, 1984a,b), it was clear that DMSO increases the affinity of the enzyme for P_i (Sakamoto, 1984a; Kandpal et al., 1987). deMeis interpreted this as an effect of DMSO on the solvation energy of P_i in medium as opposed to enzyme-bound states (deMeis, 1987, 1989). It is probable from theoretical considerations that ΔG for ATP hydrolysis in DMSO solutions is less exergonic than in aqueous solution [reviewed by deMeis (1989)], as has been seen for pyrophosphate hydrolysis (deMeis et al., 1985; Romero & deMeis, 1989). However, there is no definitive information yet for ATP hydrolysis itself (deMeis, personal communication). Also, the equilibrium between bound ATP and bound hydrolysis products (ADP and P_i) at the catalytic site, or the rates of interconversion of catalytic-site bound substrates and products, might be changed by DMSO, as entertained by Kandpal et al. (1987), who used ^{18}O-exchange methods to study these parameters.

Reversible ATP synthesis/hydrolysis, with high-affinity binding of ATP at a single site and slow release of hydrolysis products, occurs on soluble F_1 during "unisite catalysis", as discussed by Al-Shawi and Senior (1992). Using the *Escherichia coli* soluble F_1-ATPase, we have obtained each of the rate constants and equilibrium constants for the four steps of the reaction, namely, (1) ATP binding/release, (2) ATP hydrolysis/resynthesis, (3) P_i release/binding, and (4) ADP release/binding (Al-Shawi & Senior, 1992). We conceived that by measuring these parameters in the presence of DMSO, information would be obtained that would describe the effects of DMSO on the high-affinity catalytic site. Here we describe such experiments.

Materials and Methods

Materials. DMSO was obtained from Aldrich Chemical Co. (catalog no. 15493-8, ACS spectrophotometric grade). [γ-^{32}P]ATP was obtained from Amersham (catalog no. PB132). [^{3}H]ADP was obtained from NEN (catalog no. NET241). [^{32}P]P_i was obtained from Amersham (PBS13A) or ICN (enzyme-grade, catalog no. 64013). Neither [^{32}P]P_i preparation was suitable for use as supplied, since each gave considerable (≥1.5%) "leak-through" of radioactivity in Sephadex G-50 centrifuge columns in either aqueous or 40% (v/v) DMSO-containing buffers. Purification of [^{32}P]P_i was carried out by the method of Kanazawa and Boyer (1973) as abridged by deMeis and Tume (1977). After purification, the "leak-through" in centrifuge column effluents (aqueous or DMSO-containing) was reduced to ≤0.1% of applied radioactivity. Experiments were performed within 3 days of purification of the [^{32}P]P_i.

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[1] Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; ΔG_{hyd}^{ATP}, Gibbs free energy of hydrolysis of ATP; BSA, bovine serum albumin; DTT, dithiothreitol; DMSO, dimethyl sulfoxide.

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Purification and Preequilibration of F_1. Soluble F_1 was purified from strain SWM1 and stored as described by Al-Shawi and Senior (1992). Prior to each experiment, 100-μL samples of enzyme were passed through 1-mL centrifuge columns containing Sephadex G-50 in pH 7.5 buffer containing 50 mM Tris, 50 mM MOPS, 4.5 mM K_2SO_4, and 0.5 mM $MgSO_4$ adjusted to pH 7.5 with H_2SO_4. (This buffer, with or without DMSO supplementation, is the one used for all experiments and will be referred to as "pH 7.5 buffer".) This enzyme is expected from previous work to contain 3 mol of adenine nucleotide/mol of F_1 tightly bound in noncatalytic sites (Perlin et al., 1984; Issartel et al., 1986). The enzyme was then diluted with 4 volumes of the same buffer containing 50% (v/v) DMSO to give a final DMSO concentration of 40% (v/v). F_1 could be stored for 2 days in this buffer at 23 °C without loss of activity. The pH of DMSO-containing buffers was adjusted to pH 7.5 with H_2SO_4 using a glass KCl combination electrode.

Assays of Steps of Unisite Catalysis. ATP binding (k_{+1}), bound ATP hydrolysis (k_{+2}), and ATP resynthesis (k_{-2}) were measured in "acid-quench/cold-chase" experiments as described (Al-Shawi & Senior, 1992). Where 40% DMSO was present, both the F_1 and [γ-^{32}P]ATP solutions contained the organic solvent at this concentration in pH 7.5 buffer, 23 °C. The cold-chase solution contained either no DMSO, in which case the cold-chase incubation was for 2 min, or it contained 40% DMSO in which case the cold chase was for 10 min. Control experiments showed that in the latter case, this time was sufficient to convert all bound ATP to P_i. In the absence of DMSO, 96% of the added 0.2 μM [γ-^{32}P]ATP was found to be bound to F_1 and committed to hydrolysis in the cold-chase experiments, whereas in the presence of DMSO (40% v/v) this figure was 90%. Therefore, little, if any, of the bound [γ-^{32}P]ATP had entered into noncatalytic sites.

P_i Release (k_{+3}) and Equilibration of Bound ATP and Bound P_i (K_2). The methods used were as described (Al-Shawi & Senior, 1992). Briefly, 2.0 mL of F_1 solution (containing 2 mg/mL BSA) was mixed with 2.0 mL of [γ-^{32}P]ATP solution at 23 °C using vigorous magnetic stirring, and at time intervals samples were passed through centrifuge columns. Both F_1 and ATP solutions contained 40% DMSO, when present, as did the centrifuge columns.

ADP Binding (k_{-4}) and Release (k_{+4}). The centrifuge column methods of Al-Shawi and Senior (1992) were followed. Briefly, F_1 and [^{3}H]ADP were incubated together for various times and then passed through centrifuge columns, and F_1-bound radioactivity was estimated. Approximately 95% of the [^{3}H]ADP that was bound to F_1 after 30-min incubation in 40% DMSO-containing buffer was released by dilution into 40 volumes of buffer containing 5 mM ATP plus 2.5 mM $MgSO_4$ (with no DMSO), incubation for 30 s at room temperature, and elution through centrifuge columns. This showed that the [^{3}H]ADP was bound at a catalytic site.

P_i Binding (k_{-3}). [^{32}P]P_i, purified as above, was incubated with F_1 in 40% DMSO-containing pH 7.5 buffer for various times; then 100-μL aliquots were passed through Sephadex G-50 centrifuge columns containing the same buffer plus 1 mg/mL bovine serum albumin, and radioactivity in the eluates was estimated by Cerenkov counting in 0.2 M Tris (15 mL/vial) (see Table II for further details).

ATP Release (k_{-1}). This was done as described by Duncan and Senior (1985), except that here we used the pH 7.5 buffer containing 40% DMSO. It was confirmed in control experiments that hexokinase (used to convert released [γ-^{32}P]ATP to glucose 6-phosphate) was fully competent in 40% DMSO-containing buffer, pH 7.5, to trap [γ-^{32}P]ATP released.

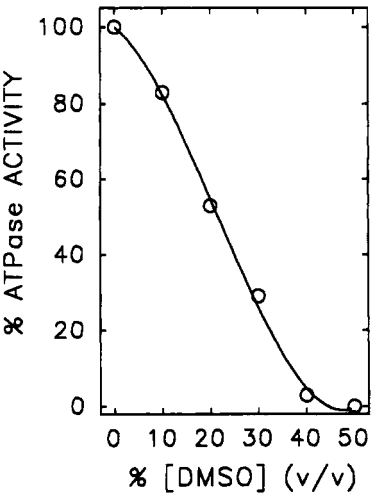

FIGURE 1: Multisite ATPase activity of *E. coli* F_1 in DMSO-containing buffers at pH 7.5. Three micrograms of F_1 was added to 1 mL of buffer (50 mM Tris–sulfate, 4.5 mM K_2SO_4, 50 mM MOPS, 2.5 mM $MgSO_4$, and 5 mM ATP, pH 7.5, containing the indicated concentrations of DMSO). Incubation was continued for 10 min at 23 °C; then 1 mL of 10% (w/v) sodium dodecyl sulfate was added, followed by 1 mL of reagent for P_i measurement (Taussky & Shorr, 1953).

Characteristics of Sephadex G-50 Centrifuge Columns Equilibrated in 40% DMSO-Containing Buffer, pH 7.5, with 1 mg/mL BSA. It was shown in control experiments using native F_1 or [^{14}C]DCCD-labeled F_1 that the recovery of F_1 protein in centrifuge column eluates under the conditions used in our experiments was routinely 75%. These columns gave the same "leak-through" of radioactive ATP, ADP, or P_i as aqueous columns.

RESULTS

Effects of Dimethyl Sulfoxide on Multisite Catalysis by E. coli Soluble F_1-ATPase. Figure 1 shows effects of DMSO on the multisite ATPase activity of F_1 at pH 7.5. There was clear, strong inhibition of activity at concentrations of DMSO >30% (v/v). However, this inhibition was rapidly reversible. When enzyme which had been equilibrated in 40% (v/v) DMSO-containing buffer was rapidly diluted by addition to 20 volumes of ATPase assay medium containing zero DMSO, full specific activity was regained within seconds. This was true even after the enzyme had stood 48 h in the 40% DMSO-containing buffer, and no evidence of enzyme precipitation was seen.

V_{max}/K_M values were determined by assaying ATPase activity in pH 7.5 buffer (with or without 40% DMSO) containing added ATP/$MgSO_4$ in a 1/1 ratio at concentrations of 0.05–10 mM. V_{max}/K_M values were 3.4×10^4 M^{-1} s^{-1} in pH 7.5 buffer alone at 23 °C and 2.68×10^2 M^{-1} s^{-1} with 40% DMSO. The difference was due entirely to changes in V_{max}; K_M was not significantly changed.

Unisite Catalysis by E. coli F_1 in DMSO-Containing Buffers. Figure 2A,B shows P_i formation rates in both "acid-quench" and "cold-chase" unisite catalysis experiments in the absence of DMSO at pH 7.5. Figure 3A,B shows the same experiment in the presence of 40% (v/v) DMSO. The rate of P_i formation in the "cold-chase" experiments is limited by the rate of binding of ATP (k_{+1}), which is seen to be significantly slower (by 42-fold) in DMSO than in aqueous buffer from comparison of plots of ln ([free F_1]/[free ATP]) (Figures 2C and 3C). The calculated k_{+1} values are shown in Table I. From the rate of approach of the curves to equilibrium in Figures 2B and 3B, the rate constants k_{+2}

Table I: Unisite Catalysis Rate Constants for *E. coli* F_1 in pH 7.5 Buffer with and without 40% (v/v) DMSO

buffer	ATP binding, k_{+1} (M^{-1} s^{-1})	ATP release, k_{-1} (s^{-1})	ATP hydrolysis, k_{+2} (s^{-1})	ATP synthesis, k_{-2} (s^{-1})	P_i release, k_{+3} (s^{-1})	P_i binding, k_{-3} (M^{-1} s^{-1})	ADP release, k_{+4} (s^{-1})	ADP binding, k_{-4} (M^{-1} s^{-1})
40% (v/v) DMSO	2.6×10^3	8.9×10^{-4}	1.3×10^{-2}	2.7×10^{-3}	7.8×10^{-4}	$\leq 7.3 \times 10^{-3}$ [a]	1.2×10^{-3}	4.0×10^1
no DMSO	1.1×10^5	2.5×10^{-5}	1.2×10^{-1}	4.3×10^{-2}	1.2×10^{-3}	4.8×10^{-4} [b]	1.6×10^{-3}	1.8×10^2

[a] Maximal value, from results of Table II. [b] Value inferred from the ΔG for ATP hydrolysis and the seven other measured rate constants. See Al-Shawi and Senior (1992) for calculation of the ΔG of ATP hydrolysis at pH 7.5, 23 °C, in the pH 7.5 buffer. As discussed in the text, significant P_i binding was not seen in aqueous buffer in direct binding assays; thus, k_{-3} could not be directly measured.

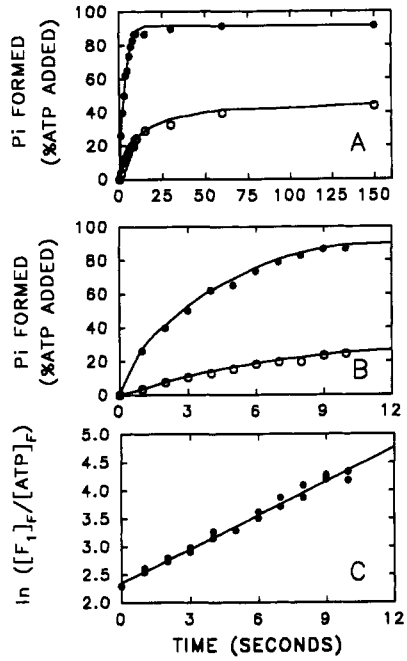

FIGURE 2: Unisite ATP hydrolysis at pH 7.5 in buffer without DMSO. (A) 12.5 μL of F_1 was mixed while vortexing with 12.5 μL of [γ-^{32}P]ATP using a 90° bent-tipped gas-tight syringe such that the final concentration ratio was 2 μM F_1/0.2 μM ATP; then it was either (a) quenched with 550 μL of pH 7.5 buffer containing 1 mM potassium phosphate, 8.2% (w/v) perchloric acid, 4.5 mM ATP, and 2.3 mM $MgSO_4$ (acid quench) or (b) diluted with 475 μL of pH 7.5 buffer containing 5 mM ATP and 2.5 mM $MgSO_4$, incubated 2 min, and then quenched with 75 μL of 60% perchloric acid and 6.8 mM potassium phosphate (cold chase). (B) Same experiments with expanded early time points. (C) Plot of ln ([free F_1]/[free ATP]) from which k_{+1} is calculated (cold-chase data only). (●) Cold chase; (○) acid quench.

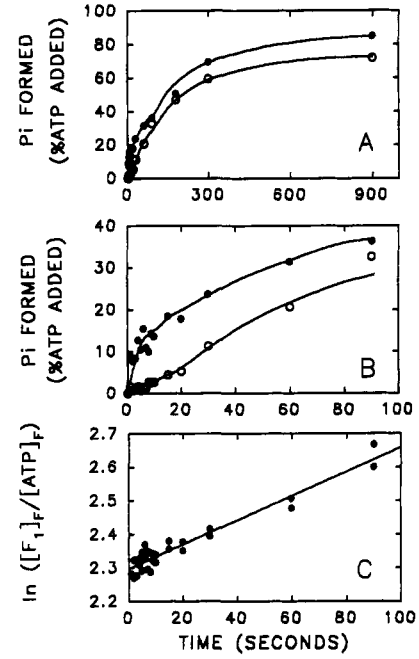

FIGURE 3: Unisite ATP hydrolysis at pH 7.5 in buffer containing 40% (v/v) DMSO. (A) The experiment is exactly the same as in Figure 2A except the F_1, [γ-^{32}P]ATP, and cold-chase solutions contained 40% (v/v) DMSO, and the cold chase was for 10 min. (B) Same as in (A) above, with expansion of the early time scale. (C) Plot of ln ([free F_1]/[free ATP]) (cold-chase data).

(hydrolysis of bound ATP) and k_{-2} (resynthesis of bound ATP from bound ADP + P_i) may be calculated (Penefsky, 1986). These rates were also slower in DMSO-containing medium by 9-fold and 16-fold, respectively (Table I). The amount of cold-chase ATP committed to hydrolysis as compared to ATP hydrolyzed in the acid-quench curve at a given time is quite small in DMSO-containing buffer in Figure 3A,B, reflective of the fact that the equilibrium constant K_2 is shifted toward bound P_i as shown in Table I.

The rate of P_i release from the catalytic sites (k_{+3}) and the equilibration of bound P_i/bound ATP were measured as described under Materials and Methods. The maximal measured fraction of the total ^{32}P that became bound to F_1 was 41% when DMSO was present, as compared to 87% when DMSO was absent, the lower value in the former case likely being due to the much reduced value of k_{+1}. The equilibrium constant K_2 (bound P_i/bound ATP) was 4.7 in the presence of DMSO, which was slightly higher than in pH 7.5 buffer alone (2.9) (Table I). The P_i release rate (k_{+3}) was 7.8×10^{-4} s^{-1} in DMSO-containing buffer, which is slightly slower than in aqueous buffer (see Table I). The rate of P_i release in DMSO-containing buffer was not affected when the experiment was repeated in the presence of 1 mM nonradioactive P_i to act as a trap. In all cases, the P_i release data were well fit by a first-order equation.

The rate of ATP release (k_{-1}) from the single high-affinity catalytic site was measured in pH 7.5 buffer with or without 40% DMSO present, as described under Materials and Methods. The rate of ATP release (k_{-1}) was found to be significantly (36-fold) slowed by DMSO (see Table I).

ADP Binding and Release. Figure 4A shows a plot of ln ([free F_1]/[free ADP]) for ADP binding to F_1 in pH 7.5 buffer containing 40% DMSO, and Figure 4B shows a plot of [ADP bound] vs time for release of ADP. The calculated values for k_{-4} (ADP binding) and k_{+4} (ADP release) in DMSO-containing buffer are shown in Table I; they are not much changed from the values in aqueous buffer and yield a K_d^{ADP} of 28.5 μM, as compared to a value of K_d^{ADP} = 8.8 μM measured at pH 7.5 in buffer with no DMSO.

P_i Binding. As noted under Materials and Methods, [^{32}P]P_i obtained from two commercial sources was found unsuitable for experiments aimed at direct measurement of P_i binding to F_1. Preliminary experiments with these unpurified preparations of [^{32}P]P_i did indeed show saturable binding of radioactivity to F_1 in DMSO-containing buffer, over and above the substantial "leak-through" seen when F_1 was omitted from the incubation mixture. This binding corresponded apparently to ~1 mol of P_i/mol of F_1 at 250 μM P_i and 2 μM F_1 concentrations in the binding incubation. Moreover, the bound radioactivity was substantially displaced (67%) by further

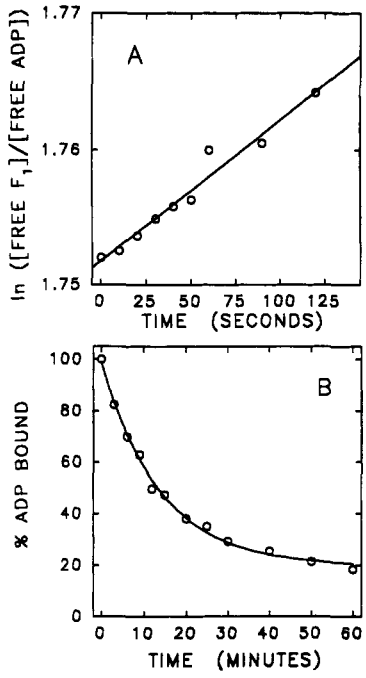

FIGURE 4: ADP binding and release. (A) ADP binding was carried out as described by Al-Shawi and Senior (1988). The concentration of F_1 was 2.62 μM, and that of [^{3}H]ADP was 0.455 μM, at 23 °C in pH 7.5 buffer with 40% DMSO (total volume = 24 μL). At the indicated times, the samples were diluted in 336 μL of pH 7.5 buffer containing 40% DMSO plus 1 mg/mL BSA, and triplicate 100-μL aliquots were passed through centrifuge columns. (B) ADP release was estimated by incubating F_1 (2.62 μM) with [^{3}H]ADP (5.7 μM) for 30 min at 23 °C in pH 7.5 buffer containing 40% DMSO. The binding mixtures (100-μL aliquots) were passed through centrifuge columns in the same buffer to separate bound from free ligand; then the eluates were diluted with 40 volumes of pH 7.5 buffer containing 40% DMSO and 1 mg/mL BSA. Aliquots (2 × 100 μL) were then passed through centrifuge columns immediately (=time zero, ADP/F_1 = 0.17 mol/mol) and subsequently at the time intervals shown. The release of [^{3}H]ADP was well fit by a first-order equation.

incubation of the centrifuge column eluates with 5 mM ATP/2.5 mM $MgSO_4$, and essentially no binding of radioactivity to F_1 was seen when a large excess (20–fold) of nonradioactive P_i was included in the incubation mixtures. However, subsequent purification of the [^{32}P]P_i virtually eliminated this binding of radioactivity, suggesting that it was due to an impurity (possibly pyrophosphate or higher polyphosphate).

Several experiments were carried out with purified [^{32}P]P_i to determine whether binding to F_1 occurred in DMSO-containing pH 7.5 buffer. In all cases, only a low level of binding occurred (0.1 mol of P_i/mol of F_1), and we cannot eliminate the possibility that the radioactivity bound was not actually P_i. A typical experiment is shown in Table II. The maximal value of P_i binding (rate constant k_{-3}) from this experiment would be $\leq 7.3 \times 10^{-3}$ M^{-1} s^{-1}, with or without sufficient ADP to saturate the first catalytic site.

Experiments were also carried out using the centrifuge column technique to determine whether purified [^{32}P]P_i bound to F_1 in pH 7.5 buffer containing no DMSO, in the presence or absence of added $MgSO_4$ or ADP (2.5 mM). No significant P_i binding was seen. Further, in a buffer consisting of 40 mM Tris–acetate/1 mM $MgSO_4$, pH 6.5 [as used by Penefsky (1977) and Kasahara and Penefsky (1978)], with 1mM [^{32}P]P_i and 2 μM F_1, in quadruplicate experiments the amount of radioactivity bound corresponded to only 0.125 P_i/F_1 (mol/mol). No binding of radioactivity occurred with purified F_1–α- or F_1–β-subunits alone, and binding of radioactivity to F_1 was abolished by incubation on ice, indicating that an oligomeric F_1 was required. However, we cannot rule out the possibility that the radioactivity bound could be pyrophosphate or a polyphosphate impurity, rather than phosphate itself.

Therefore, the major conclusion from these experiments is that *E. coli* F_1 does not display significant P_i binding as measurable by the centrifuge column technique either in aqueous buffer at pH 6.5–7.5 or in 40% DMSO-containing buffer at pH 7.5. It should be noted that given our calculated dissociation rate constant (k_{+3}) for P_i dissociating from the high-affinity catalytic site of 7.8×10^{-4} s^{-1} (with DMSO) or 1.2×10^{-3} s^{-1} (without DMSO) (Table I), it can be concluded that if P_i did, in fact, bind to the high-affinity catalytic site, it should not have been lost from that site during the centrifuge column elution procedure.

Equilibrium Constants for Unisite Catalysis in 40% Dimethyl Sulfoxide Containing Buffer at pH 7.5. Table III presents the equilibrium constants for unisite catalysis in 40% DMSO buffer, pH 7.5, as compared to the same buffer without DMSO.

Table II: Does [^{32}P]P_i Bind to *E. coli* F_1 in DMSO-Containing Buffer?[a]

incubation conditions	P_i bound/F_1
F_1 + P_i	0.074
F_1 + P_i + ADP	0.050

[a] F_1 (6.05 μM) and [^{32}P]P_i (982.5 μM, 270.5 dpm/pmol, purified as described under Materials and Methods) were incubated together in a total volume of 80 μL in 40% (v/v) DMSO, 50 mM Tris–sulfate, 50 mM MOPS, 4.5 mM K_2SO_4, and 0.5 mM $MgSO_4$, pH 7.5, for 3 h at 23 °C. Where ADP was present, it was added to F_1 10 min before the addition of P_i (300 μM for 10 min with F_1 alone and then 150 μM for 3 h with P_i plus F_1). At the end of the 3-h incubation, 20 μL of DMSO-containing buffer which also contained 1 mg/mL bovine serum albumin was added, and the whole 100-μL sample was passed through a 1-mL Sephadex G-50 centrifuge column (Penefsky, 1977) equilibrated in DMSO-containing buffer, pH 7.5, plus 1 mg/mL BSA. Quadruplicate determinations were recorded. In this experiment, the centrifuge column "leak-through" (with no F_1) was 0.098% of the dpm with no ADP present and 0.094% of the dpm with ADP present.

Table III: Equilibrium Constants of Unisite Catalysis in DMSO-Containing or Aqueous Buffer at pH 7.5

buffer	K_a^{ATP} (K_1) (M^{-1})	K_2	$K_d^{P_i}$ (K_3) (M)	K_d^{ADP} (K_4) (M)
40% DMSO	2.9×10^6	4.7	0.11[a]	2.85×10^{-5}
no DMSO	4.4×10^9	2.9	2.4[b]	8.8×10^{-6}

[a] Minimal value, based on values for k_{+3}/k_{-3} in Table I. [b] Values based on inferred value of k_{-3} as described in Table I footnote *b*.

Discussion

In the first part of this work, we showed that DMSO markedly inhibits multisite (cooperative) catalysis in *E. coli* F_1-ATPase. V_{max}/K_M was reduced by 2 orders of magnitude in 40% (v/v) DMSO, primarily due to inhibition of V without significant change of K_M. These effects were rapidly reversed on dilution into aqueous buffer. K_M is thought to be reflective of binding of ATP at the third catalytic site during multisite (cooperative) ATP hydrolysis, i.e., not the site of actual bond cleavage (Boyer, 1989). Therefore, these results show that the primary effect of DMSO is at the first (unisite) catalytic site. The third site is expected from its apparent lower affinity to be a more open (hydrophilic) site than the first site (Al-Shawi et al., 1990b), and the results are consistent with this idea.

Measurement of the binding of substoichiometric ATP at a single catalytic site using techniques for assay of unisite catalysis demonstrated that the ATP binding rate was substantially decreased in 40% DMSO-containing buffer (Table I). The release of ATP from this site was also significantly

faster, resulting in a decrease in K_a^{ATP} by 3 orders of magnitude (Table III). This suggests that the driving force for binding and sequestration of ATP in normal enzyme at the high-affinity catalytic site derives considerably from the hydrophobic nature of the catalytic site as compared to aqueous medium. This is consistent with previous suggestions that the F_1-ATPase high-affinity catalytic site presents a hydrophobic, highly constraining environment for ATP binding (deMeis, 1989; Al-Shawi et al., 1990a,b). It is noteworthy that K_d^{ADP} was changed by only 3.2-fold under the same conditions, suggesting a significant difference between the conformation of the high-affinity catalytic site for ADP binding as opposed to ATP binding, with the ADP-binding conformation likely to be the more hydrophilic. Our results for ADP binding are consistent with those of Sakamoto (1984a), who found only slightly decreased ADP binding to beef heart mitochondrial F_1 in the presence of 35% (w/v) DMSO-containing buffer.

The equilibrium constant (K_2) for interconversion of bound ATP and bound ADP + P_i at the high-affinity catalytic site was 4.7 in 40% DMSO as compared to 2.9 in aqueous buffer (Table III). This is not a large effect. On the other hand, both the forward and backward rate constants (for ATP hydrolysis and resynthesis, k_{+2} and k_{-2}) were slowed considerably (by 9-fold and 16-fold) by DMSO. This effect may be rationalized by the hypothesis (Al-Shawi et al., 1990a,b) that reaction rates at the first catalytic site are dependent on binding energy derived from tight, specific binding of the substrates and products and that the reduction in binding affinity for ATP (and presumably, ADP + P_i) is reflected in a reduction in the catalysis rate. The large reduction in V_{max} during multisite catalysis brought about by 40% DMSO (above) may also be largely explicable on this basis.

Kandpal et al. (1987) previously noted similar effects of DMSO on mitochondrial F_1, using completely different methods of analysis, namely, measurements of medium $P_i \rightleftharpoons H^{18}OH$ exchange and dependence of the formation of enzyme-bound ATP on P_i concentration. Notably, these authors found the equilibrium constant (K_{-2}) governing reversible formation of bound ATP from bound ADP and P_i was around unity in DMSO medium (as in aqueous media) but that the rates of interconversion of bound substrates and products (k_{+2} and k_{-2}) were lowered by 10–20-fold in DMSO-containing as compared to aqueous media. The agreement between the different experimental approaches (oxygen isotope exchange and pre-steady-state unisite kinetic measurements) confirms that the characteristics of the same high-affinity catalytic site are being measured.

Previous workers have concluded that a major effect of DMSO is to increase the affinity for P_i at the catalytic site (Sakamoto & Tonomura, 1983, 1984a,b; Kandpal et al., 1987; deMeis, 1987, 1989). We were unable to confirm this directly by experimental measurement, since the association rate constant for binding of P_i to *E. coli* F_1 in 40% DMSO-containing buffer was $\leq 7.3 \times 10^{-3}$ M^{-1} s^{-1} in the presence or absence of sufficient ADP to saturate the high-affinity site (Table II). An effect of DMSO on P_i binding in *E. coli* F_1 is therefore not apparent, in contrast to mitochondrial F_1 (Sakamoto, 1984a). However, it is appropriate to note that in aqueous buffer mitochondrial F_1 appears to bind P_i more tightly at the catalytic site than *E. coli* F_1 does. For example, at pH 8.5 the inferred $K_d^{P_i}$ values were reported to be 78 and 2300 M, respectively (Al-Shawi et al., 1989).

DMSO is known to significantly alter the ΔG for pyrophosphate hydrolysis, in a positive direction (Romero & deMeis, 1989), and it is reasonable to anticipate that ATP hydrolysis may also be less exergonic in DMSO solutions than in aqueous buffers (deMeis, 1989), although no definitive data on this point are currently available (deMeis, personal communication). Using the measured values for K_1, K_2, and K_4 in 40% DMSO from Table III, together with the estimated minimal value for K_3 of 0.11 M in 40% DMSO from Table III, one may calculate an overall minimal K_{app} for ATP hydrolysis in 40% DMSO at pH 7.5 of 42, as compared to 2.7×10^5 in aqueous buffer at pH 7.5. Thus, the ΔG for ATP hydrolysis in 40% DMSO-containing buffer must be ≥ -9.2 kJ/mol. If we make the assumption that K_3 is not likely to be >2.4 M in DMSO-containing buffer at pH 7.5 (since that is the calculated value for K_3 in aqueous medium, pH 7.5), then the ΔG for ATP hydrolysis in 40% DMSO would be maximally −16.8 kJ/mol, i.e., considerably less than the value of −30.7 kJ/mol for ATP hydrolysis at pH 7.5 in aqueous buffer of similar constitution (Al-Shawi & Senior, 1992).

Registry No. ATPase, 9000-83-3; DMSO, 67-68-5; ATP, 56-65-5; ADP, 58-64-0; P_i, 14265-44-2.

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Activation of Methotrexate-α-Alanine by Carboxypeptidase A-Monoclonal Antibody Conjugate†

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ABSTRACT: Carboxypeptidase A (CP-A) and monoclonal antibody KS1/4 directed against an antigen on human lung adenocarcinoma cells (UCLA-P3) were derivatized by treatment with succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate and *N*-succinimidyl 3-(2-pyridyldithio)propionate, respectively. The derivatized proteins were reacted to produce thioether-linked enzyme-antibody conjugates. Sequential HPLC size-exclusion and DEAE chromatography separated the conjugate preparation from unreacted enzyme and antibody. On the basis of SDS-PAGE analysis and measurement of catalytic activity, the preparation contained approximately equal amounts of 1:1 and 2:1 (enzyme:antibody) conjugates; binding activity of the conjugate (1.8×10^5 molecules/cell) was similar to that of unreacted antibody. In vitro cytotoxicity studies with UCLA-P3 cells demonstrated the ability of cell-bound conjugate to convert the prodrug methotrexate-α-alanine (MTX-Ala) to methotrexate (MTX). In the absence of conjugate, ID_{50} values for MTX-Ala and MTX were 8.9×10^{-6} and 5.2×10^{-8} M, respectively. ID_{50} for the prodrug improved to 1.5×10^{-6} M with cells containing bound conjugate. This potentiation of MTX-Ala cytotoxicity by conjugate-bound CP-A, which was at least 30-fold greater than that produced by a comparable amount of free enzyme, is attributed to enhanced effectiveness of MTX generated at the cell surface as opposed to the surrounding medium. Examination of the time course of cytotoxicity over a 96-h period showed that the conjugate-prodrug combination (at 2.5×10^{-6} M) was nearly as effective as MTX in preventing cell replication. These results demonstrate the chemotherapeutic potential of carboxypeptidase-monoclonal antibody conjugates used in conjunction with MTX peptide prodrugs.

Activation of prodrugs by enzyme-monoclonal antibody conjugates, which is a novel and potentially very useful strategy in cancer chemotherapy, provides a mechanism for the *selective* delivery of drugs to tumor sites [reviewed by Bagshawe (1987); Bagshawe, 1989; Senter, 1990]. Methotrexate α-peptides (i.e., derivatives in which amino acids are linked covalently to the α-carboxyl group of MTX[1]) are prodrug forms of the parent antifolate. These compounds are much less toxic to cells than MTX, presumably because of their inability to be internalized by folate transport systems (Sirotnak et al., 1979). MTX peptides can be activated, however, by carboxypeptidase-mediated hydrolysis to yield the parent drug (Kuefner et al., 1989). Regional selectivity of the activation process has been demonstrated in a model system in which L1210 mouse leukemia cells and methotrexate-α-alanine (MTX-Ala) were dispersed in soft agarose and carboxypeptidase A (CP-A) was immobilized on beads in the center of the plate; under these conditions, a zone of cell kill was observed radiating outward from the beads (Vitols et al., 1989).

The present investigation was undertaken to extend the studies with MTX-Ala by determining whether cytotoxic concentrations of MTX could be produced by the amount of CP-A that could be attached to target cells via a monoclonal antibody. For this purpose, CP-A was conjugated to KS1/4, a monoclonal antibody directed toward a cell surface glycoprotein associated with a variety of epithelial carcinomas

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[1] Abbreviations: MTX, methotrexate; MTX-Ala, methotrexate-α-alanine; SMCC, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); CP-A, carboxypeptidase A; PBS, phosphate-buffered saline; DMF, dimethylformamide; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorter; ID_{50}, drug concentration for 50% inhibition of growth; rt, retention time.

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