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ACTIVATION ENERGIES FOR THE ATP-DRIVEN REVERSAL OF OXIDATIVE PHOSPHORYLATION IN SUBMITOCHONDRIAL PARTICLES

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SUMMARY

The participation of energy transfer Factor B at the second phosphorylation site between cytochromes *b* and c_1 in the respiratory chain was tested spectroscopically at the wavelength pair 562–575 mμ with ascorbate *plus* *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) as substrate and ATP as energy source for the reversal of oxidative phosphorylation. At temperatures above 30°, Factor B produced a distinct stimulation of the activity of ammonia particles but did not alter the extent of cytochrome *b* reduction. Below 30° no effect of Factor B could be observed. A similar though not completely matching biphasic thermal effect was also seen in the reversal of oxidative phosphorylation between NADH dehydrogenase and cytochrome *b* (site 1) promoted by Factor A or B with the urea and ammonia–EDTA particles, respectively. A phosphorylating electron transport particle from heavy mitochondria (ETP_H) showed the same biphasic thermal response in the ATP-driven NAD^+ reduction as well as in its oligomycin-sensitive ATPase activity without the addition of any factors.

In all the above cases, there was a break in the Arrhenius plot around 30° except for the reversal of oxidative phosphorylation at site 1 in ETP_H. The activation energies and their changes on supplementation with the energy transfer factors differed for the particles. Factor B had no effect on the activation energy of ammonia particles in the reversal of site 1 or 2 but simply increased the rate of the reversal. The effect of oligomycin at low levels in the reversal of site 1 phosphorylation with ammonia particles was similar to the effect of Factor B. It is concluded, in agreement with previous reports, that oligomycin and Factor B act in the same region of the energy transfer reactions. Factor A strikingly decreased the activation energy for the reversal of phosphorylation at site 1 below 31° in urea particles from 43.8 to 24.3 kcal/mole, supporting a structural role for

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; ETP_H, phosphorylating electron-transport particles from heavy layer bovine heart mitochondria; PCMS, *p*-chloromercuriphenylsulfonate.

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the factor in stimulating oxidative phosphorylation. Above 31° the change was from 27.5 to 20.5 kcal/mole.

Omission of dithiothreitol or addition of low levels of *p*-chloromercuriphenylsulfonate (PCMS) produced a decrease in the rate of reversal of site 1 phosphorylation in ETP_H. Concomitant with this decrease a break was induced in the Arrhenius plot at about 28°. The activation energy above the break was between 11.8 and 12.8 kcal/mole, similar to the value for Factor B-supplemented ammonia particles in the same temperature range. Below the transition temperature, the activation energy was 24.6–26.2 kcal/mole.

It is concluded from these studies that reconstitution of oxidative phosphorylation occurs best at temperatures between 30 and 43° possibly due to easier reassembly of the membrane structure at the higher temperatures.

INTRODUCTION

Several energy transfer factors of oxidative phosphorylation have been tested from the stand point of their participation at more than one phosphorylation site of the respiratory chain. Site 1 specificity is tested by the increase of the glucose-6-^{32}P/NADH ratio obtained in the oxidation of NADH by terminally inhibited submitochondrial particles with coenzyme Q_1 (refs. 1–3) or fumarate[4] as electron acceptors, or stimulation of the ATP-dependent succinate-linked NAD^+ reduction[5–11]. A way of measuring site 2 specificity was suggested by TYLER *et al.*[12], involving the influence of purified energy transfer factors on the ATP-dependent reduction of cytochrome *b* by ascorbate–TMPD. This possibility was tested in the present study. Surprisingly, no detectable stimulation could be produced by Factor B at temperatures below 30° whereas strong stimulation was observed above 30°. This prompted us to carry out a systematic study of the effect of temperature on the reversal of oxidative phosphorylation and on the action of different energy transfer factors in the reconstitution of this activity in depleted submitochondrial particles. The results of the experiments in the thermodynamic characterization of such a reconstitution are presented in this paper. Furthermore, an attempt has been made to identify thermodynamically one of the steps involved in energy transfer in a phosphorylating electron-transport particle (ETP_H) from heavy mitochondria by inhibitor studies and to compare it with the same step in reconstituted systems in order to test whether the isolated energy transfer factor is indeed a component of the phosphorylating system.

MATERIALS AND METHODS

Energy transfer factors and assay particles

Factor B was prepared according to LAM *et al.*[9] with modifications[13,14]. The 10 mM Tris–H_2SO_4 fraction from CM-cellulose was used in our assays. Its specific activity was generally around 2.0 (ref. 9) but sometimes the values were between 5.0 and 6.0.

Factor A was prepared according to WARSHAW *et al.*[15] using the modification

introduced by SANI *et al.*[16]. The 80 mM peak from DEAE-cellulose[16] was used in our experiments.

ETP_H was prepared according to LINNANE AND ZIEGLER[17] and ammonia particles as reported by LAM *et al.*[9]. The urea particles were made according to ANDREOLI *et al.*[8] using ETP_H as starting material and incubating it for 30 min at 5 mg particle protein per ml in a medium containing 0.25 M sucrose–1 M urea. The pellet after centrifugation and rinsing with sucrose was suspended in 0.25 M sucrose–10 mM Tris–H_2SO_4 (pH 7.5) to a particle concentration of 25–30 mg protein per ml for storage at —70°.

Assay methods

ATP-supported cytochrome *b* reduction was performed in a reaction medium containing 0.16 M sucrose, 25 mM triethanolamine–H_2SO_4 (pH 7.8), 2.7 mM $MgCl_2$, 0.54 mg bovine serum albumin per ml, 1.7 mM dithiothreitol, submitochondrial particles and energy transfer factors in concentrations as indicated in RESULTS. After incubation for 2 min at the desired temperature, the following were added successively at intervals of 15 sec: ATP (2 mM), ascorbate (15 mM), TMPD (50 μM), and finally KCN (0.5 mM) to start the reaction. The initial activity was determined from the slope of the absorbance change over the period between 15 and 60 sec after addition of KCN. When succinate (6.5 mM) was then added, further reduction of cytochrome *b*, which was inaccessible to ascorbate–TMPD, was achieved. The reaction was recorded at 562–575 mμ* as described by TYLER *et al.*[12] in a cuvette maintained at the preincubation temperature in the Aminco–Chance dual-wavelength spectrophotometer.

ATP-driven NAD^+ reduction by succinate was measured essentially as described by LAM *et al.*[13] under procedure B in the presence of dithiothreitol (5 μmoles per 2.9 ml preincubation mixture). The concentration of submitochondrial particles was 0.5 mg protein per 3 ml assay mixture unless otherwise specified. Triethanolamine–acetate (pH 7.8) was the buffer component instead of Tris–H_2SO_4 since pH of Tris buffer varies considerably with temperature[18]. Initial activity after addition of KCN was determined at indicated above, the assay extending over a period of 2–4 min in addition to a 2-min preincubation period at the desired temperature. Specific activity of energy transfer factors is expressed as defined before[9].

ATPase activity was measured according to LAM AND YANG[19] at pH 7.6 in triethanolamine–acetate buffer. Concentrations of ETP_H, $MgCl_2$ and ATP were 0.2 mg, 2 and 10 μmoles, respectively, in a total volume of 1 ml. The duration of the assay was 5 min following a 2-min preincubation period at the desired temperature.

The protein concentration of mitochondrial and submitochondrial particle preparations was determined by a biuret method[20] using bovine serum albumin as a standard. Protein concentrations in the energy transfer factor preparations were determined according to LOWRY *et al.*[21] using the same standard.

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* It was demonstrated by measuring absorption spectra at liquid N_2 temperatures that the 562–575-mμ absorbance change in the type of experiments to be reported here is due to reduction of cytochrome *b* (ref. 12). Although such demonstration was not made specifically in the present experiments, it will be assumed that this absorbance change is a measure of cytochrome *b* reduction.

RESULTS

During an investigation of the effect of energy transfer factors on the energy-dependent cytochrome *b* reduction by ascorbate–TMPD in depleted submitochondrial particles, the temperature of the reaction mixture was lowered. This was done in order to make the rates of reduction more easily measurable, and to make it feasible to trap the partially completed reaction in liquid N_2 for spectroscopically relating the increased rate of 562–575-mμ change to cytochrome *b* reduction (Fig. 1a) (refs. 12, 22). It turned out, however, that hardly any stimulation of the rate was produced by Factor B at temperatures below 25° (Fig. 1a), the stimulation appeared around 30° and reached a maximum between 41 and 43°. It was observed earlier that the extent of cytochrome *b* reduction in certain particles by ascorbate–TMPD was incomplete since succinate produced further reduction[12]. It may be seen in Fig. 1a that Factor B hardly affected the extent of this reduction, leaving the difference between cytochrome *b* reduction by ascorbate–TMPD and that by succinate[12] unchanged.

Fig. 1b gives the activation energies for the experiment shown in Fig. 1a. A break occurred in the Arrhenius plots for the particle in the presence or absence of added Factor B, respectively, at 32–33.2 and 37.4–42.2° (range for two experiments). It is evident from the data that Factor B had little effect on the activation energies of the particle above and below the break. The values were 15.4–17.1 and 4.2–6.6 kcal/mole, respectively. In the presence of Factor B these values were 13.2–14.9 and 6.6–7.7 kcal/mole, respectively.

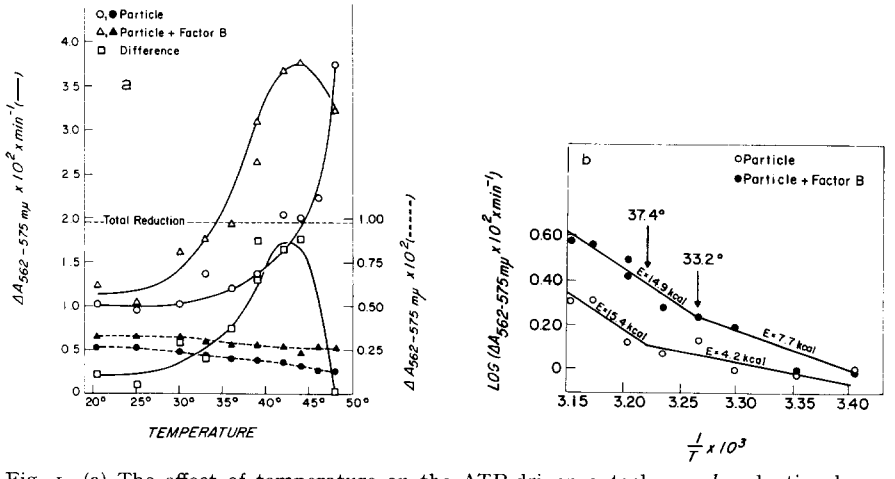

Fig. 1. (a) The effect of temperature on the ATP-driven cytochrome *b* reduction by ascorbate *plus* TMPD and on its stimulation by Factor B. The standard assay conditions are described under MATERIALS AND METHODS. Ammonia particles were used in the concentration of 2.33 mg protein in 3 ml reaction volume. The amount of Factor B used in the experiment (29 μg/mg particle protein) saturated the same particle in the ATP-driven NAD^+ reduction with succinate as substrate at 38°. The specific activity of Factor B in that assay was 4.2 μmoles NADH · mg protein $^{-1}$ · min^{-1}. Indicated in the figure on the left is the initial activity ($\Delta A_{562-575\ m\mu} \times 10^2$ min^{-1}, open symbols), and the extent of reduction ($\Delta A_{562-575\ m\mu} \times 10^2$, closed symbols) is on the right ordinate. Total reduction was measured by subsequent addition of succinate as described in MATERIALS AND METHODS. (b) Activation energies (*E*) for ATP-driven cytochrome *b* reduction. The values in kcal/mole were computed from data of (a). *T* is the absolute temperature.

A similar effect of temperature on Factor B activity as described above is also evident in the ATP-dependent NAD^+ reduction by succinate (Figs. 2a and 3a). The specific activities of both Factor A (ref. 8) and Factor B are uniformly small below 28–30° and increase from 30 to 43°, thereby also showing a biphasic stimulatory characteristic.

The Arrhenius plots (Figs. 2b and 3b) reveal interesting differences, however, although a break occurs in each plot. Similar to the situation involving the reversal of site 2 phosphorylation (Fig. 1b), Factor B did not significantly change the activation energy of the ammonia particle on either side of the temperature break

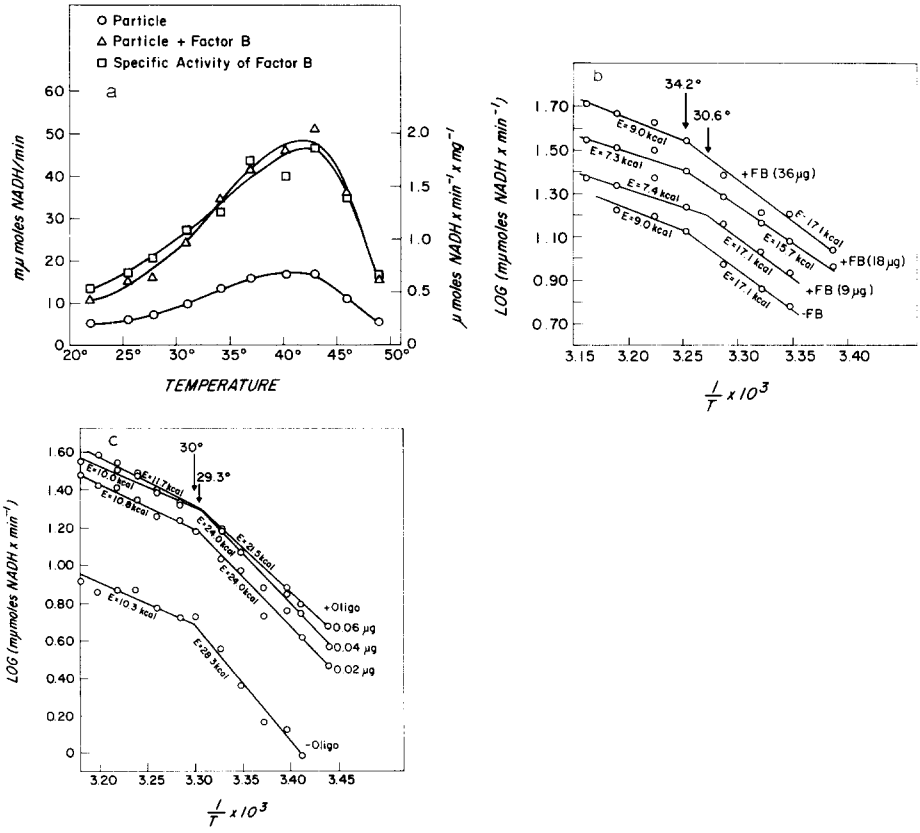

Fig. 2. (a) Stimulation by Factor B of the ATP-driven NAD^+-reduction activity as a function of temperature. Ammonia particles were used in the experiment, the pH of the reaction medium being 7.7. Succinate was the electron donor. Factor B concentration was 36 μg/mg particle protein. For the determination of the specific activity of Factor B (μmoles $NADH \cdot min^{-1} \cdot mg\ protein^{-1}$, □) three levels of Factor B were used, *viz.* 9, 18 and 36 μg/mg particle protein, at each temperature. (b) Arrhenius plots for the ATP-driven NAD^+ reduction. The data shown in (a) were used for this plot. The data for particles alone (–FB) and for particles in the presence of three different levels of Factor B (+FB) expressed as μg factor per mg particle protein are shown in the figure. (c) Arrhenius plots for the ATP-driven NAD^+ reduction by succinate with ammonia particles in the absence and presence of oligomycin. The particles were preincubated for 2 min with oligomycin (0.02, 0.04 and 0.06 μg/mg particle protein) in the reaction mixture before the NAD^+ reduction was started by the addition of KCN. Oligomycin was dissolved in ethanol and 0.01, 0.02 and 0.03 ml was added to the preincubation mixture (total reaction volume 3 ml); pH of the medium was 7.7. The ordinate of the figure indicates the log of the initial activity.

in the reversal of phosphorylation at site 1, but simply increased the rate of NAD^+ reduction (Fig. 2b). The breakpoint occured at 30.6–34.2° with values of 7.3–9 and 15.7–17.1 kcal/mole above and below the temperature break, respectively, in the presence or absence of the factor. A duplicate set of experiments at pH 7.8 showed a breakpoint of 28° and activation energies of 10.6–12.7 and 15.7–18 kcal/mole above and below that temperature, respectively. In this respect, the effects of Factor B and oligomycin are quite similar as can be seen from Fig. 2c. Using a different ammonia particle preparation from the one in Fig. 2b, a break occured at 30.1° with activation energies of 10.3 and 28.3 kcal/mole above and below that temperature,

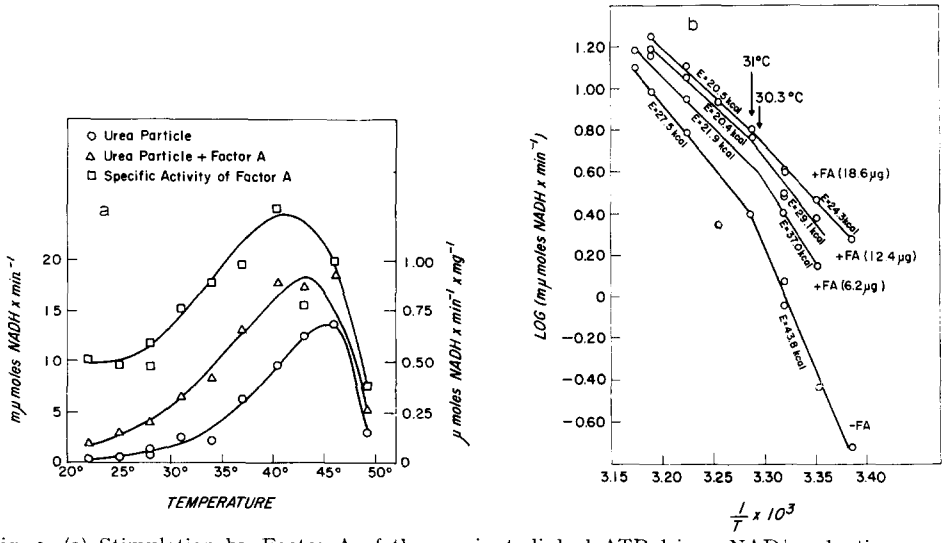

Fig. 3. (a) Stimulation by Factor A of the succinate-linked ATP-driven NAD^+ reduction as a function of temperature. The initial activity is expressed as $m\mu$moles $NADH \cdot min^{-1}$. The triangles represent a Factor A concentration of 18.6 μg/mg urea particle protein. For determination of the specific activity of Factor A (μmoles $NADH \cdot min^{-1} \cdot mg$ $protein^{-1}$, □) three different levels were used (6.2, 12.4 and 18.6 μg/mg particle protein) at each temperature. (b) The effect of Factor A on the activation energies. Indicated are data for particles alone (–FA) and with Factor A (+FA) expressed as μg factor per mg particle protein. The data in (a) are represented by the top and bottom lines.

respectively. Activation energies in the presence of oligomycin were 10–11.7 and 21.5–24 kcal/mole, respectively, above and below a similar temperature break (29.3°, Fig. 2c).

This contrasts with the effect of Factor A in the same assay with urea-treated particles (Fig. 3b). The particle itself showed an unusually high activation energy of 43.8 kcal/mole at temperatures below the breakpoint of 31° and an activation energy of 27.5 kcal/mole above it (Fig. 3b). Factor A gradually lowered the activation energy of both segments of the Arrhenius plot to 24.3 and 20.5 kcal/mole, respectively.

It was of interest to compare the activation energy characteristics obtained with depleted and reconstituted particles[9] with that derived from a undepleted competent particle, ETP_H[17]. The comparison is particularly relevant with one of the depleted particles (urea particles) since it is derived from ETP_H (see MATERIALS AND METHODS). Fig. 4a shows the relationship between succinate-linked ATP-dependent

NAD^+ reduction activity and temperature. In contrast to the results with the urea-depleted particles (Fig. 3b), ETP_H showed no break in the Arrhenius plot between 20 and 38° (Fig. 4b). A straight line representing an activation energy of 23.1 kcal/mole could be drawn through the experimental points.

The effect of assay temperature on the Mg^{2+}-dependent oligomycin-sensitive ATPase activity of ETP_H is shown in Fig. 5a. The release of P_i from ATP by ETP_H is oligomycin sensitive over the temperature range between 16 and 50°. Above 50° the oligomycin sensitivity decreases, possibly as a result of an irreversible conformational change in the terminal phosphorylation enzyme from the Factor-A form to the F_1-type aberrant ATPase conformation[15]. An Arrhenius plot of the oligomycin-sensitive ATPase (triangles of Fig. 5a) shows a discontinuity at 34.4–35.2° with acti-

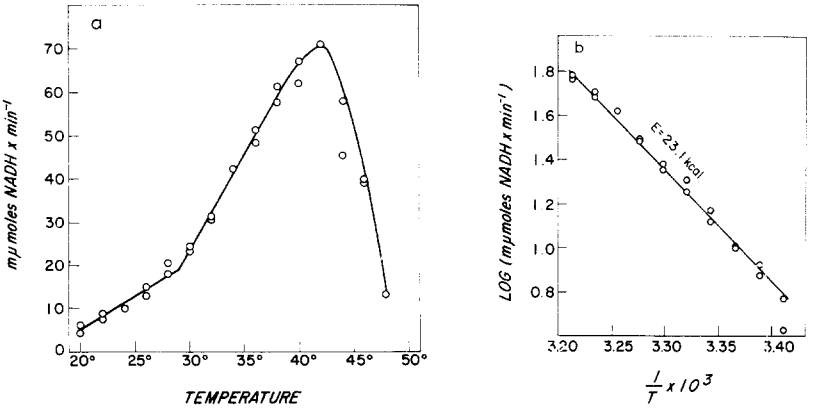

Fig. 4. (a) ATP-driven NAD^+ reduction by succinate as a function of temperature. The ordinate indicates initial activity of NAD^+ reduction. ETP_H concentration was 0.82 mg protein per 3 ml total reaction volume. (b) Arrhenius plot for the ATP-driven NAD^+ reduction by succinate using ETP_H. The temperature range in the experiment was from 20 to 38°. The data in (a) were used for this plot.

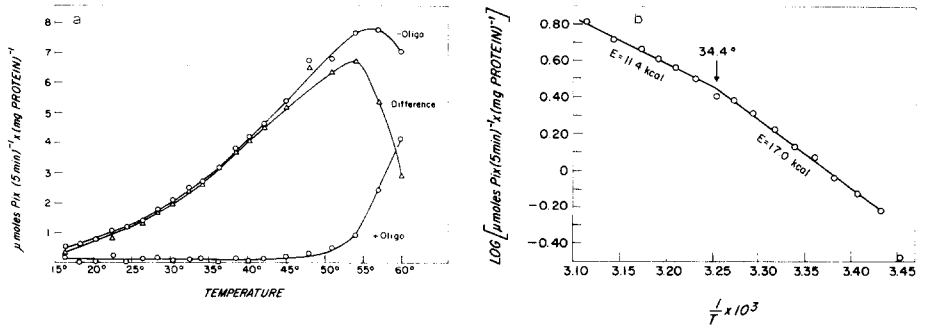

Fig. 5. (a) Mg^{2+}-dependent oligomycin-sensitive ATPase activity of ETP_H as a function of temperature. In one series of experiments oligomycin was present at a concentration of 5 μg/mg particle protein in the preincubation mixture and in another simultaneous series oligomycin was omitted. The oligomycin-sensitive ATPase is taken as the difference between these two values. Stock solution (1 mg/ml) of oligomycin was prepared in 95% ethanol and diluted with water (1:100) immediately before use. (b) Arrhenius plot of the oligomycin-sensitive ATPase of ETP_H. The values were computed from the data in (a).

vation energies of 9.3–11.4 kcal/mole above this thermal break and 16.6–17 kcal/mole below it (Fig. 5b).

Comparison of Figs. 3b and 4b shows that the activation energy characteristics of urea particles supplemented with Factor A approach those of the "parent" particle ETP_H. This is especially true when one considers the fact that the highest level of Factor A used in Fig. 3b was not saturating at temperatures above 31°, while it was below 31°. In all these data, the greatest similarity is between the ammonia particles, with or without Factor B (Fig. 2b) and ETP_H in the Mg^{2+}-dependent oligomycin-sensitive ATPase (Fig. 5b). The Arrhenius plots are quite similar, and the activation energy for the higher temperatures is about one-half of that for the energy-linked NAD^+ reduction by ETP_H (Fig. 4b).

In a final experiment the effect of low (1 and 2 μM) levels of PCMS on the ATP-driven succinate-linked NAD^+ reduction by ETP_H was investigated. These low levels have little effect on electron transport between succinate or NADH and O_2 but make some process in the energy-transfer pathway rate-limiting[23]. It can be seen that the Arrhenius plot for the uninhibited reaction in the presence of dithiothreitol is a straight line as in Fig. 4b, representing an activation energy of 19.7 kcal/mole (Fig. 6). Omission of dithiothreitol not only decreased the reduction rates but also induced a break in the Arrhenius plot at 27.4° and changed the activation energy both above and below the break. The activation energy above the break was lowered to 12.8 kcal/mole, a level comparable to that of the Factor B-controlled reaction in ammonia particles at temperatures above a similar discontinuity in the Arrhenius plot (7.3–12.7 kcal/mole, Fig. 2b). The activation energy below the break was increased

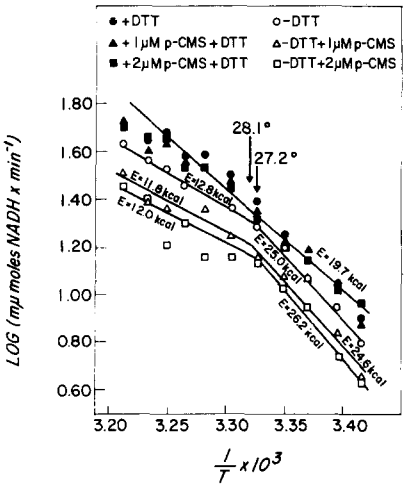

Fig. 6. Arrhenius plots for the ATP-driven NAD^+ reduction by ETP_H. Succinate was the substrate in the presence or absence of dithiothreitol and PCMS. ●, + dithiothreitol; ▲, + 1 μM PCMS + dithiothreitol; ■, + 2 μM PCMS + dithiothreitol; ○, − dithiothreitol; △, − dithiothreitol + 1 μM PCMS; □, − dithiothreitol + 2 μM PCMS. The PCMS was added to the particles prior to the addition of the other components of the reaction medium. The logarithm of maximal activity of NAD^+ reduction is given on the ordinate. The two sets of solid points at the upper part of the graph (*i.e.* $1/T \times 10^3$ around 3.22) appear below the line drawn through the remaining points. This may be because over the 9-h period of experimentation, the ETP_H had declined in activity. Similar points appeared on the line in other experiments.

to 25 kcal/mole, a value incompatible with the action of Factor B in a comparable temperature range (16–18 kcal/mole, Fig. 2b). Addition of PCMS caused a further decrease in the rates of NAD^+ reduction but did not alter the position of the temperature break, nor the activation energies below and above the break (Fig. 6). It can be seen from the same figure that addition of dithiothreitol abolished the effect of PCMS.

DISCUSSION

Site specificity of Factor B

From the studies of LAM *et al.*[9], it is apparent that Factor B stimulates oxidative phosphorylation not only at the first phosphorylation site (*i.e.* between the NADH dehydrogenase flavoprotein and cytochrome *b*), but also at one or both the sites between cytochrome *b* and O_2. In the present study, the stimulatory effect on the second phosphorylation site, *i.e.* between cytochrome *b* and c_1 (refs. 12,22) has been demonstrated (Fig. 1a).

Factor B, however, did not increase the extent of cytochrome *b* reduction, leaving unaltered the difference in extent of reduction by ascorbate–TMPD and by succinate. In tightly coupled mitochondria all of the cytochrome *b* is reduced by ascorbate *plus* TMPD in the presence of ATP, since there is no further change in the 562–575-mμ absorbance on the subsequent addition of succinate[12,22]. The latter reduces cytochrome *b* in a non-energy-linked manner[12]. It is apparent from this that Factor B alone is not able to restore the interaction of cytochrome *b* with the energy transfer enzymes once the communication was lost during the preparation of submitochondrial particles.

The Arrhenius plots for the reversal of oxidative phosphorylation at sites 1 and 2 catalyzed by the ammonia particle show distinct breaks (Figs. 1b, 2b) whether Factor B is present or not. Factor B stimulates both reactions but does not significantly affect the activation energies on either side of the break. Since both reactions respond similarly to Factor B, the conclusion that it acts at the level of the non-phosphorylated high-energy intermediate[24] appears consistent. The actual values for the activation energies are, however, different for reversal of sites 1 and 2 (*cf.* Figs. 1b and 2b). Presumably this difference could be related to the differing segments of the respiratory chain involved in the two reactions.

Activation energies and role of energy transfer factors

The activation energy of 43.8 kcal/mole for the reversal of site 1 phosphorylation in urea particles below 31° (Fig. 3b) is unusually high compared to other particles and may indicate a structural rearrangement in the particle. This value compares favourably with the high activation energy (37.8–38.6 kcal/mole) observed by TRAUB AND NOMURA[25] for the reconstitution of ribosomes in the temperature range of 20–40°. Since the rate-limiting step was accompanied by a distinctly positive entropy change, they interpreted the results as an opening up or loosening of the structure. In a similar manner, the effect of Factor A in lowering the activation energy to almost half its value (Fig. 3b) could mean that Factor A has a role in restoring the integrity of the membrane besides a catalytic role in stimulating oxida-

tive phosphorylation. This was recognized for F_1 earlier by SCHATZ *et al.*[26] and PENEFSKI[27]. It may be pointed out that Factor A and F_1 are functionally indistinguishable in restoring oxidative phosphorylation and appear to be different conformational forms of the same ADP phosphorylation enzyme[9,15].

Factor B (Figs. 1b and 2b) in contrast to Factor A (Fig. 3b) produces virtually no alteration in the activation energy of the ammonia particle depleted of the complementary factor. One possible reason for the difference may be the fact that this particle is not completely depleted of Factor B. Its presence and participation in the reaction is shown by the inhibition of the basal energy-linked activities by a serum immune to Factor B (anti-Factor B)[19], and by the stimulation of the basal activities by low levels of oligomycin. The oligomycin appears to act by stabilizing the high-energy intermediates[28–30], making the endogenous Factor B functionally more effective. This explanation is consistent with the observation that oligomycin also does not alter the activation energies (Fig. 2c).

The striking similarity in activation energy characteristics of the Factor B-controlled reaction in ammonia particles (Fig. 2b) and the Mg^{2+}-dependent oligomycin-sensitive ATPase of ETP_H (Fig. 5b) does not necessarily mean that Factor B is involved in the latter reaction. In fact, this similarity is most probably coincidental, since the oligomycin-sensitive ATPase was only marginally inhibited by preincubation of ETP_H with $1 \cdot 10^{-4}$ M PCMS in accordance with the results of LAM AND YANG[19] (but in disagreement with those of TZAGOLOFF *et al.*[31] who obtained 50% inhibition with $1 \cdot 10^{-4}$ M *p*-chloromercuribenzoate on a purified oligomycin-sensitive ATPase complex from beef heart mitochondria). Treatment of Factor B with PCMS on the other hand inhibits the activity[13,32]. Therefore, the action of Factor B has been localized outside the oligomycin-sensitive ATPase segment[24].

Factor B is apparently one of the components made rate-limiting in ETP_H by omission of dithiothreitol or addition of low levels of PCMS in the absence of dithiothreitol as a consequence of an effect on the functional –SH group of Factor B (refs. 13, 32). Activation energies above 27.2–28.1° (11.8–12.8 kcal/mole, Fig. 6) fall in the range observed for the action of Factor B on ammonia particles in a similar temperature range (7.3–12.7 kcal/mole, Fig. 2b). This is consistent with our interpretation that Factor B exerts its function also in the undepleted ETP_H and is thus no isolation artifact but a factor genuinely involved in energy transfer. The present data give no clue, however, whether the action of Factor B on energy transfer is direct or indirect. Activation energies at temperatures below the break in Fig. 6 (24.6–26.2 kcal/mole) are incompatible with those of the Factor B-stimulated reaction below a similar break in the Arrhenius plot (16–18 kcal/mole, Fig. 2b). This may be due to another dithiothreitol and mercurial-sensitive step in energy transfer besides the one in which Factor B is rate-limiting, as postulated by SANADI *et al.*[24] on the basis of inhibitor studies with PCMS.

Inhibitor studies such as those described above may be useful for the thermodynamic characterization of other steps in oxidative phosphorylation. By comparison of the activation energies of inhibitor-induced rate-limiting steps with those of factor-depleted and factor-stimulated reactions it might be possible to localize the site of action of the energy transfer factors. Other reagents that could be used in this manner are oligomycin and uncouplers. As inhibitors specific for the energy transfer factors one might use their antibodies[2,19].

Activation energy as a criterion for satisfactory reconstitution

Comparison of Arrhenius plots using submitochondrial particles depleted of energy transfer factor in the presence and absence of their complementary factors with the plots for the same energy-linked process catalyzed by the undepleted relatively intact ETP_H may be useful in assessing the effectiveness of the reconstitution of the complex oxidative phosphorylation system. However, this tool can be used more safely as a negative criterion. For instance, the striking difference between the activation energy characteristics of the energy-linked NAD^+ reduction by ammonia particles in the presence of saturating levels of Factor B (Fig. 2b) and by ETP_H (Fig. 4b) under the same conditions can be attributed to incomplete reconstitution. There must be some other altered functional or structural disarrangement which is not repaired by Factor B. On the other hand, the resemblance of the Arrhenius plots of urea-treated particles with increasing Factor A supplementation (Fig. 3b) and of ETP_H (Fig. 4b) does not mean that reconstitution is entirely complete since it is known[9] that Factor B will produce further stimulation of the reaction. Complete restoration of both activity and of the activation energy pattern comparable to that of ETP_H might be a better criterion for sound reconstitution of the integrated membrane system.

Biphasic thermal response of reversal of oxidative phosphorylation

Biphasic thermal response in activity *versus* temperature has been observed with all the submitochondrial particles we have tested (Figs. 1–5), with a change in slope near 30°. However, consideration of the activation energies and other data as discussed above do not point to any single common step or principle underlying this phenomenon. Thus, it is at present impossible to decide whether a given break in an Arrhenius plot indicated some conformational change of a membrane component, or merely a change in the rate-limiting step. The latter explanation probably does hold for the break in Fig. 6 (see DISCUSSION under *Activation energies and role of energy transfer factors*). Nor is it known whether such a behavior also applies for the forward reactions of oxidative phosphorylation. KEMP *et al.*[33] found a biphasic thermal response in some of the forward reactions of oxidative phosphorylation catalyzed by rat liver mitochondria between 6 and 30° with a break in the Arrhenius plot at 16–18°. Most of the reactions were apparently controlled by the rate-limiting adenine nucleotide translocase. Other reactions could be controlled by transport phenomena as well so that no conclusion can be made from their data as to the effect of temperature on oxidative phosphorylation as such.

GENERAL CONCLUSIONS

It should be admitted that the reversal of oxidative phosphorylation in submitochondrial particles is a complex system involving several reactions, some of which may be unknown at present. It is thus impossible to make firm conclusions from the measurements on activation energies reported in this paper. The results, however, allow a tentative conclusion that both conformational changes in the membrane and changes in the rate-limiting step of the process may be involved in producing the observed changes in Arrhenius plots and activation energies. In

addition, it should be recognized that the kinetics of the different steps in reversed electron transfer may conceivably change independently of each other as the temperature is varied and, as an extreme situation, the observed activation energies may even represent composite values whose interpretation may be ambiguous.

A result of paramount importance emerging from our studies is that reconstitution studied *via* the reversal of oxidative phosphorylation is best investigated at temperatures between 30 and 43°. This range falls within the region in which TRAUB AND NOMURA[25], although working with a totally different membrane system got best reconstitution of 30-S ribosomes from 16-S ribosomal RNA and 30-S ribosomal proteins. It appears that energy transfer factors bind better above 30°, and indeed we found some evidence for this in the experiments with urea particles and Factor A. Levels of Factor A that apparently saturated the particle activity at or below 31° did not do so between 31 and 40.5°, although this may also mean, as emphasized above, that different steps in the reaction process were rate-limiting in the different temperature ranges. Experiments designed to investigate the binding of energy transfer factors to complementary deficient particles as a function of temperature are now in progress.

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