Phosphate Dependence and Atractyloside Inhibition of Mitochondrial Oxidative Phosphorylation

THE ADP-ATP CARRIER IS RATE-LIMITING*

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ATP production and oxygen consumption by rat liver mitochondria were measured as a function of atractyloside and phosphate (Pi) concentration. The "on" kinetics of atractyloside inhibition of ATP production are very rapid, and the onset of inhibitory effect is complete within 1 s even at concentrations which produce partial inhibition. A ligand conservation plot relating atractyloside concentration and fractional inhibition of ATP production is linear and indicates that inhibition is proportional to the fraction of ADP-ATP carrier sites bound with atractyloside. Estimates of atractyloside inhibition constant, Kd, are 3.8 \times 10^{-7} \text{ mol/g of protein} and 2.2 \times 10^{-8} \text{ M}, respectively.

Mitochondrial respiration during active oxidative phosphorylation is proportional to log [Pi]. Plots of atractyloside concentration versus respiratory rate at different Pi concentrations are similar in shape. There is no increase in sigmoidicity with decreasing Pi that would suggest that the ADP-ATP carrier is losing any rate-limiting character as Pi decreases and the rate of the reaction falls.

Hexokinase at concentrations below about 400 units/g of mitochondrial protein limits the rate of mitochondrial respiration in the presence of glucose and ATP. Atractyloside inhibition curves become increasingly sigmoidal as hexokinase is decreased below 400 units/g of protein. This finding demonstrates that when the ADP-ATP carrier is not rate-limiting, significant amounts of atractyloside can bind without producing a corresponding decrease in ATP production.

It is concluded that, in rat liver mitochondria, adenine nucleotide translocation is rate-limiting in the overall reaction of oxidative phosphorylation and is responsible for the phenomenon of respiratory control.

It is well known that mitochondrial respiration coupled to oxidative phosphorylation is stimulated by ADP (1). The ratio of respiration in the presence of excess exogenous ADP to respiration in its absence is the respiratory control ratio. The mechanism underlying the phenomenon of respiratory control is in dispute. Heldt and Klingenberg (2) have proposed that the ADP-ATP exchange carrier, Pi still exerts a marked and undiminished effect upon the rate of oxidative phosphorylation, at least at temperatures below 23°C (cf also Ref. 3). Since ATP competes with ADP for the exchange carrier, respiration coupled to ATP synthesis is, therefore, controlled by the ratio of ADP to ATP as has been reported (4, 5). Moreover, because ADP-ATP exchange is largely electrogenic, i.e. ADP\(^{-}\) is exchanged for ATP\(^{+}\), the electrical potential across the inner membrane is an additional factor controlling the rate of exchange (6, 7).

Wilson and his collaborators (8-10) have shown that respiration coupled to ATP synthesis is proportional not only to the ADP to ATP ratio but also to inorganic phosphate (Pi) concentration. Since Pi does not participate in the ADP-ATP exchange reaction and had not previously been reported to affect significantly the rate of adenine nucleotide exchange (11), Wilson and co-workers (8-10) conclude that the ADP-ATP exchange carrier is not rate-limiting in oxidative phosphorylation and is not responsible for the phenomenon of respiratory control. They proposed instead that the actual rate-limiting step is the reaction(s) with molecular oxygen and that this reaction is in near equilibrium with the phosphorylation of ADP in such a manner that electron flux is proportional to log [ATP]/([ADP][Pi]).

In this communication, we confirm that respiration is proportional to Pi concentration in tightly coupled rat liver mitochondria. However, we present evidence that the ADP-ATP exchange carrier is, nevertheless, rate-limiting over a 16-fold range of Pi concentration. Moreover, when the ADP-ATP carrier is made unequivocally rate-limiting by partially inhibitory concentrations of atractyloside, a specific inhibitor of the ADP-ATP exchange carrier, Pi, still exerts a marked and undiminished effect upon the rate of oxidative phosphorylation. These data are interpreted in terms of the relative contributions of the pH gradient and the membrane potential to the protonmotive force across the inner membrane and their effects on the rate of adenine nucleotide translocation.

**MATERIALS AND METHODS**

Mitochondria from rat liver were isolated in 0.25 M sucrose (12) and routinely gave respiratory control ratios of 5 to 8 after addition of 300 M ADP to a medium containing 150 M sucrose, 5 M MgCl\(_2\), 5 M disodium succinate, 5 M rotenone, 1 mg/ml of mitochondrial protein, 10 M KP, buffer, pH 7.4, 23°C. Oxygen was measured polarographically and ATP was measured continuously with firefly luciferase luminescence as previously described (13-15). The first derivative of the oxygen signal was generated by an electrical circuit similar to that described by Sargent and Taylor (16). Protein was determined by a biuret procedure (17) using bovine serum albumin as standard. Purified firefly luciferase and synthetic firefly luciferin were obtained from DuPont Corp. Adenine nucleotides, atractyloside, and crystalline yeast hexokinase were obtained from Sigma (St. Louis, Mo.).
RESULTS

Atractyloside is a specific inhibitor of adenine nucleotide transport through the inner mitochondrial membrane. In order to assess the extent to which the ADP-ATP exchange carrier is rate-limiting in oxidative phosphorylation, atractyloside inhibition of active mitochondrial ATP synthesis was examined using the luminescence technique (Fig. 1). The onset of atractyloside inhibition of ATP production occurs immediately (within a stirring time of approximately 1 s) even at concentrations which only partially inhibit. The ATP concentration at the end of each recording in Fig. 1 was determined after the addition of ATP standard. From these values was subtracted the ATP concentration at the end of the 8 μM atractyloside recording in which inhibition was presumed to be 100%. This difference was divided by the time interval between inhibitor addition and the end of the rising portion of the recording or the end of the recording, whichever came first. The result is the rate of ATP production at each atractyloside concentration. From instantaneous oxygen electrode recordings corresponding rates of oxygen consumption were calculated for each atractyloside concentration. There is a close correspondence between inhibition of ATP production and inhibition of respiration (Fig. 2A). The ATP/O ratio is only 1.03 in the absence of inhibitor since almost 90% of the added nucleotide was AMP.

The plot of atractyloside concentration versus the rate of mitochondrial ATP production (Fig. 2A) shows a slight degree of sigmoidicity at low atractyloside concentrations. This small shoulder on the left part of the curve is a consequence of atractyloside binding to the ADP-ATP carrier and of the resulting decrease in free atractyloside concentration. The ligand conservation plot is useful in determining the inhibition constant (K_i) and the concentration of inhibitory sites (E) when K_i is less than or equal to E (18, 19). The ligand conservation plot is based upon the equation:

\[
\frac{[I]}{[i]} = \frac{K_i}{(1 - i)} + E
\]

where \([I]\) is inhibitor concentration and \(i\) is fractional inhibition. By this equation, a plot of \(1/(1 - i)\) against \([I]/[i]\) forms a straight line with a slope of \(K_i\) and a y-intercept of \(E\). The rates of ATP production at different atractyloside concentrations are plotted in this fashion in Fig. 2B. From the slope and intercept of the line fitting the data points, the number of atractyloside-sensitive sites (E) is \(3.8 \times 10^{-7}\) mol/g of mitochondrial protein, and the atractyloside inhibition constant (K_i) is \(2.2 \times 10^{-8}\) mol. In the ligand conservation plot, it is assumed that the fractional inhibition of the activity being measured equals the fraction of inhibitor sites with bound inhibitor. The linearity of the ligand conservation plot in these experiments suggests, therefore, that the fractional inhibition of mitochondrial ATP production during oxidative phosphorylation equals the fraction of ADP-ATP carriers bound with atractyloside. This condition should obtain only if the ADP-ATP carrier is a rate-limiting step in oxidative phosphorylation. If the translocase is not in fact rate-limiting, then it must be very nearly rate-limiting since partial inhibition can be detected even when the concentration of inhibitory sites exceeds atractyloside concentration by as much as 3.8-fold.

Wilson and co-workers (8-10) have reported that respiration coupled to oxidative phosphorylation is proportional to log [Pi]. They conclude from this observation that the ADP-ATP carrier is not a rate-limiting step in oxidative phosphorylation. We observe a similar dependence on Pi in our mitochondrial preparations (Fig. 3A). As Pi concentration decreases, so does the State 3 respiratory rate. At 0.5 mM Pi, the rate is 64% of the rate at 8 mM Pi. In the presence of partially inhibitory amounts of atractyloside, Pi retains its influence on respiratory rate (Fig. 3, B and C). Plots of atractyloside concentration versus maximal State 3 respiratory rate at different Pi concentrations are similar in shape (Fig. 4): there is no increase in sigmoidicity as Pi decreases. Such an increase in sigmoidicity would suggest that significant amounts of atractyloside are binding to the ADP-ATP carriers without producing a corresponding decrease in ATP production, i.e. that the ADP-ATP carrier is losing its rate-limiting character as the overall rate of the reaction falls. The concentration of atractyloside causing 50% inhibition of oxidative phosphorylation is greater in these experiments than in the previous experiments (Fig. 2).
because much greater concentrations of exogenous adenine nucleotide (4.5 mM) were employed. For any inhibition by atracyloside of net oxidative phosphorylation to occur, ADP-ATP translocation must become rate-limiting. Since P_i stimulates respiration even in the presence of partially inhibitory concentrations of atracyloside, one cannot use this P_i dependence as evidence that ADP-ATP translocation is not a rate-limiting step in oxidative phosphorylation.

We argue that the lack of increasing sigmoidicity in the atracyloside inhibition curves with decreasing P_i supports our contention that the ADP-ATP carrier remains rate-limiting as P_i is varied. As a positive control, we examined atracyloside inhibition of oxidative phosphorylation under conditions where the ADP-ATP exchange reaction is not rate-limiting. The exchange reaction was made non-rate-limiting by employing glucose and hexokinase to stimulate respiration and control respiratory rate. In the presence of saturating glucose (5 mM) and ATP (300 μM), respiratory rate is dependent upon hexokinase concentration up to about 400 units of hexokinase/g of mitochondrial protein (Fig. 5). We examined atracyloside inhibition of hexokinase-stimulated respiration at different concentrations of hexokinase (Fig. 6). At excess hexokinase (600 units of hexokinase/g of mitochondrial protein), there is a slight sigmoidicity of the inhibition curve. As hexokinase concentration decreases, the inhibition curves become progressively more sigmoidal. This is exactly the expected result if, at low hexokinase concentrations, the ADP-ATP carrier is no longer rate-limiting in oxidative phosphorylation.

**DISCUSSION**

The kinetic data of atracyloside inhibition are fully consistent with carrier-mediated ADP-ATP translocation being a rate-limiting step in oxidative phosphorylation. If the ADP-ATP carrier were not rate-limiting, then atracyloside should be able to occupy a significant fraction of the total ADP-ATP carrier binding sites without causing an inhibition of the overall rate of oxidative phosphorylation. However, even when atracyloside binding sites were in 3.8-fold excess over total atracyloside concentration, significant (16%) inhibition of ATP production by oxidative phosphorylation was observed. Values of the inhibition constant (K_i) and the concentration of inhibitory sites (E) as estimated from a linear ligand conservation plot were $2.2 \times 10^{-8}$ M and $3.8 \times 10^{-7}$ mol/g of protein, respectively. These values are in agreement with direct determinations of high affinity atracyloside binding sites and of the K_i of such binding (20, 21).

The dependence of mitochondrial respiration on P_i concentration has been cited by Wilson and co-workers (10) as evidence that ADP-ATP translocation is not a rate-limiting step in oxidative phosphorylation. However, we are able to demonstrate such P_i dependence even when net oxidative phosphorylation is partially inhibited by atracyloside. Since the ADP-ATP carrier must be rate-limiting under such circumstances, the question arises as to how P_i exerts its effect.

One consequence of the electrogenic nature of ADP^3--ATP^4-- exchange is that the rate of exchange is strongly dependent upon membrane potential: an increase in negative membrane potential increases the rate of exchange of extramitochondrial ADP^3-- for intramitochondrial ATP^4-- (6, 20). According to Mitchell's chemiosmotic hypothesis (22), the
membrane potential and the pH gradient across the membrane together constitute the protonmotive force which is the driving force for ATP synthesis. Recent studies have shown that increasing Pi concentration decreases the pH gradient contribution to the protonmotive force (7, 23). If we assume that there is a compensatory increase in membrane potential such that the protonmotive force remains unchanged, then increasing Pi concentration should increase the rate of exchange of ADP for ATP and thereby augment the overall rate of oxidative phosphorylation.

Another consequence of the electrogenic nature of the ADP-ATP exchange reaction is a relative concentration of ADP extramitochondrially and ATP intramitochondrially if there is a negative membrane potential (7, 24). The electroneutral Pi-OH exchange reaction is important as well since it catalyzes the accumulation of Pi if there is a positive pH gradient (25). Thus, in the presence of a negative membrane potential and a positive pH gradient, i.e. the two components of the protonmotive force, the ratio [ATP]/[ADP][Pi] will be greater outside than inside the mitochondrion. This ratio can be expressed logarithmically as the Gibbs free energy, ΔGp, for ATP synthesis from ADP and Pi, also known as the phosphate potential (26). Phosphate potentials generated extramitochondrially are 14.6 to 16.2 kcal/mol, while those generated intramitochondrially are 10.4 to 11.8 kcal/mol (4, 24, 26). The importance of the Pi and adenine nucleotide exchange systems in raising the extramitochondrial ΔGp is underscored by the observation that homogeneously inverted inner membrane vesicles prepared by sonication generate a ΔGp of 9.8 to 11.4 kcal/mol which is similar to the ΔGp generated internally by mitochondria (13, 14, 27, 28). Collectively the two transport systems act to dissipate the protonmotive force. However, the energy expended is not lost but is conserved as an increased phosphate potential in the extramitochondrial space. Therefore, we conclude that the ADP-ATP carrier is not only responsible for the phenomenon of respiratory control but is also responsible together with the Pi carrier for the elevated ΔGp of the extramitochondrial space.

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