Absence of a Common Intermediate Pool among Individual Enzyme Chains of the Energy-Conservation Pathway in Chloroplasts of *Euglena gracilis*

By JOSEPH S. KAHN

Department of Biochemistry, North Carolina State University, Raleigh, N.C. 27607, U.S.A.

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Tri-*n*-butyltin chloride is a specific inhibitor that binds stoichiometrically and irreversibly with the ATP-synthesizing sites of chloroplasts. Titration of *Euglena* chloroplasts with tri-*n*-butyltin chloride shows about six ATP-synthesizing sites per molecule of cytochrome c_{552} or per 380 molecules of chlorophyll. This system was used to study the possibility of linkage between individual enzyme chains of the energy-conservation pathway or the possible existence of a common pool of an intermediate in this pathway. The inhibition of ATP synthesis by tri-*n*-butyltin chloride at low rates of electron transport (low light-intensities, NADP⁺ or ferricyanide as electron acceptor) agrees with a kinetic model of two to four ATP-synthesizing sites per energy-conservation chain. At high rates of electron transport, however, the results occasionally agree with a model of 20 or more sites per chain. The results are interpreted as indicating the absence of a common intermediate pool, but the presence of a limited degree of linkage between individual chains. It also indicates the presence of two energy-conservation sites in these chloroplasts.

The concept of the minimal size of a chloroplast fragment retaining its ability to catalyse the Hill reaction has undergone a number of transformations (Emerson & Arnold, 1932; Park & Pon, 1961; Duysens, 1966). From recent work it has become evident that different segments of the overall reaction sequence require different minimal sizes for their function. According to Izawa & Good (1965), approx. 2500 molecules of chlorophyll are present for each oxygen-evolving site, implying one site for a number of electron-carrier chains.

I have tried to determine a similar minimum unit for the energy-conservation pathway in chloroplasts. I have used TBT* to determine the possible existence of linkage between, or a common intermediate to, a number of individual enzyme chains of the pathway. By the terms 'enzyme chain' or 'energy-conservation chain' used in this text, I mean an organized system of enzymes of a pathway arranged morphologically so as to favour a sequence of binary reactions between its adjacent components to the exclusion of similar components in neighbouring chains. TBT is a highly specific inhibitor of ATP synthesis in chloroplasts, binding stoichiometrically and irreversibly to a terminal site in the energy-conservation pathway close to the site of action of the terminal coupling factor (CF₁) (Kahn, 1968*a,b*). The effect of TBT on

chloroplasts is similar to that of oligomycin on mitochondria.

EXPERIMENTAL

The culturing of cells and isolation of chloroplasts have been previously described (Kahn, 1966) and have been modified as follows (I.C. Chang & J. S. Kahn, unpublished work): cells were grown at 25–27°C with 1.1% (v/v) ethanol as carbon source. They were illuminated at 3000 lx with cool white fluorescent light.

The cells were harvested at a population density of 6×10^6 – 8×10^6 cells/ml (10–15 h after the end of the exponential phase of growth). The grinding medium was 0.4 M-mannitol, 25 mM-tris-HCl buffer, pH 8.0, and 10 mM-NaCl. The cell suspension was rapidly frozen in a low-temperature bath to facilitate breakage, thawed, homogenized with one-eighth the volume of 3–5 mm polyethylene pellets in a chilled Waring Blendor for 2–3 min and filtered through cheesecloth to remove the beads. The filtrate was centrifuged at 1500 g for 1 min to remove intact cells, cell debris and paramylum granules from the mixture. The supernatant was then centrifuged at 1500 g for 10 min to sediment whole chloroplasts, which were resuspended in the grinding medium and resedimented by centrifugation at 1000 g for 10 min. The washed chloroplasts were finally suspended in the grinding medium with the addition of 10 mg of bovine serum albumin/ml.

Photophosphorylation experiments were carried out as previously described (Kahn, 1966, 1968*a*), with the variations as described in the figures.

* Abbreviation: TBT, tri-*n*-butyltin chloride.

TBT was freshly dissolved daily in 50% (v/v) methanol, and all reactions contained a final concentration of 3% (v/v) methanol.

Radioactive TBT, labelled with ^{113}Sn , was synthesized by Dr William P. Tucker (Tucker, 1968). ^{113}Sn is a commercially available weak γ -emitter, with half-life 118 days.

For studies on the binding of [^{113}Sn]TBT, the chloroplasts were suspended in a medium containing 0.5M-mannitol, 50mM-tris-HCl and 10mM-NaCl. The inhibitor was added in a minimum volume of methanol, and the chloroplasts were reisolated immediately, either by rapid centrifugation or by adding 4 times their volume of 50% (v/v) ethanol followed by centrifugation. The pellet, as well as the supernatant, was assayed in a Nuclear-Chicago model 4216 γ -counter with an efficiency of approx. 8%.

THEORY

Under conditions of saturating light-intensity with pyocyanine as a cofactor for cyclic photophosphorylation, electron transport is at a maximum and the rate of ATP synthesis is limited by the terminal steps in the energy-conservation pathway (Kahn, 1968a; also see Fig. 2). Under these conditions the rate of ATP synthesis decreases linearly with increasing [TBT]/[chlorophyll] ratios, i.e. the rate depends on the number of uninhibited sites.

When the rate of electron transport is decreased by lowering the light-intensity, or when NADP⁺ or ferricyanide is used as electron acceptor for non-cyclic photophosphorylation, one can set up two boundary cases for the effect of TBT on the rate of ATP synthesis as follows. (1) If each energy-conservation chain has only a single ATP-synthesizing site at its disposal, the number of active sites would continue to be the rate-limiting factor for ATP synthesis at any given rate of electron transport. Consequently the rate of ATP synthesis would decrease linearly with increasing [TBT]/[chlorophyll] ratios, regardless of the initial uninhibited rate. (2) If each energy-conservation chain has free access to all ATP-synthesizing sites, i.e. one of the intermediates serves as a common pool, then the rate of electron transport will be rate-limiting for ATP synthesis when light-intensity is decreased. The addition of TBT would not affect the rate of ATP synthesis so long as the combined activity of all uninhibited ATP-synthesizing sites was in excess of the rate of intermediate formation at the site of energy conservation. In Fig. 2 one would obtain a line parallel to the abscissa to a point at which the ATP-synthesizing sites become rate-limiting. This would be the point of intersection with the diagonal line on the figure.

Any model that stipulates a finite number of ATP-synthesizing sites per energy-conservation chain ($1 < n < \infty$) will yield a family of curves of ATP synthesis versus [TBT]/[chlorophyll] ratio based on the binomial distribution of the number of inhibited ATP-synthesizing sites per chain at any given ratio of inhibitor addition. The basic minimum assumption underlying these calculations is that either all energy conservation chains have to be equally active, or that all these chains have identical reactivities towards TBT. Another assumption is that the inactivation of one site by TBT affects neither the activity nor the reactivity of adjacent sites.

If there are m levels of inhibitor addition, then at any

level t ($t = 0, 1, 2, \dots, m$), the fraction of all sites inhibited is p_t , and the limiting conditions are:

when $t = 0$, no inhibition, $p_0 = 0$

when $t = m$, full inhibition, $p_m = 1$

The total number of ATP-synthesizing sites per energy-conservation chain is n , a positive integer; the number of inhibited sites, $k = 0, 1, 2, \dots, n$.

One can thus define the expression for a binomial distribution such that at inhibitor level t the fraction of the total energy-conservation chains having k sites inhibited is:

$$p_t(k) = \binom{n}{k} p_t^k (1 - p_t)^{n-k}$$

This expression yields a matrix P with $m + 1$ rows and $n + 1$ columns, where p_t is increased linearly as follows: $p_0 = 0$, $p_1 = 1/m$, $p_2 = 2/m$, ..., $p_m = m/m = 1$, and, in general, $p_{t+1} = p_t + p_1$.

What this matrix provides is the relative distribution at any given level of inhibitor addition of energy-conservation chains having 0, 1, 2, ..., n of their ATP-synthesizing sites inhibited. This is a binomial distribution table, and the values of $p_t(k)$ for values of n ranging from 2 to 20 were taken from Burington & May (1953).

We now set up a vector, $S = (s_0, s_1, s_2, \dots, s_n)$, having $n + 1$ components. Taking the potential fractional activity, s_k , of any energy-conservation chain having k of its sites inhibited, the vector is set in the following way:

$$S = \left(s_0 = \frac{n}{n}, s_1 = \frac{n-1}{n}, s_2 = \frac{n-2}{n}, \dots, s_n = \frac{n-n}{n} \right)$$

bound by the condition:

$$\frac{n-k}{n} \leq \frac{a}{A_{\max.}}$$

where a is the uninhibited rate of ATP synthesis at lowered rate of electron transport and $A_{\max.}$ is the maximal rate of ATP synthesis obtained in full light and with pyocyanine as cofactor.

One now multiplies componentwise every row of the P matrix starting with $t = 0$ by the vector S for the same value of n and the desired value for $a/A_{\max.}$ and adds up the products. This yields the ordinate values for the theoretical curve on the graphs of ATP synthesis versus [TBT]/[chlorophyll] ratio.

RESULTS

Stoichiometry of TBT binding to chloroplasts. The stoichiometric binding of TBT was determined in two independent ways. Chloroplasts titrated with [^{113}Sn]TBT showed saturation of binding sites at [TBT]/[chlorophyll] 0.016–0.017, i.e. one molecule of TBT to 60 molecules of chlorophyll (Fig. 1). Similar values were obtained when ATP synthesis in chloroplasts was plotted against the [TBT]/[chlorophyll] ratio (Fig. 2) and extrapolated to zero activity.

Fig. 1. Binding of $[^{113}\text{Sn}]\text{TBT}$ by chloroplasts. The reaction was carried out as described in the Experimental section. ●, Treated chloroplasts isolated by centrifugation ×, treated chloroplasts diluted to 4 times their volume with 50% (v/v) ethanol, followed by centrifugation. ----, Line of 100% binding.

Fig. 2. Effect of TBT on ATP synthesis by chloroplasts under saturating light-intensity. The reaction mixture, in a final volume of 1.5 ml, contained: $15 \mu\text{mol}$ of tris-HCl buffer, pH 7.8, $50 \mu\text{mol}$ of NaCl, $5 \mu\text{mol}$ of MgCl_2 , $4 \mu\text{mol}$ of ADP, $4 \mu\text{mol}$ of P_i containing 5×10^5 c.p.m. of ^{32}P , $0.05 \mu\text{mol}$ of pyocyanine, 1 mg of crystalline bovine serum albumin, chloroplasts containing $20 \mu\text{g}$ of chlorophyll and 3% (v/v) methanol. Illumination (105000 lx) was for 2 min at 17°C .

The latter method, with saturating light-intensity and pyocyanine as cofactor, was used as a routine to determine the maximal rate of ATP synthesis (A_{max}) as well as the point of total inhibition (p_m). The two values were determined by application of the method of least squares. Up to the point of saturation, i.e. $[\text{TBT}]/[\text{chlorophyll}]$ 0.016, all the TBT added was bound to the chloroplasts, as determined by removing the chloroplasts by centrifugation and adding fresh chloroplasts to the supernatant (see also Kahn, 1968a).

Taking 380 as the number of chlorophyll molecules

per cytochrome c_{552} , b_{556} or b_{563} in *Euglena* (Perini, Kamen, & Schiff, 1964; Ikegami, Katoh, & Takamiya, 1968), six TBT-binding sites or ATP-synthesizing sites per electron-carrier chain are obtained. This does not imply six energy-conservation sites ($\text{P}/2e^- \neq 6$), since all six TBT-binding sites could conceivably be bound to a single energy-conservation chain.

Effect of TBT on ATP synthesis under conditions of decreased illumination. When the rate of electron transport is lowered by diminished light-intensity, the decrease in ATP formation with increased additions of TBT ceases to be linear. The activity approaches zero, however, at the same $[\text{TBT}]/[\text{chlorophyll}]$ ratio that is required for complete inhibition of ATP formation under conditions of saturating light-intensity.

To determine the number of ATP-synthesizing sites (n) that would be required for each energy-conservation pathway to account for the non-linearity, I have calculated theoretical curves corresponding to different values of n and superimposed them on the experimental points. For initial rates (a/A_{max}) less than 60%, a model corresponding to $n = 2-4$ always gives the best agreement (Figs. 3a and 3b). For initial rates greater than 60%, the best fit was usually for $n = 2-4$, except for a number of cases (one in three or four) where a model of $n = 20$ or more gave the best agreement (Figs. 3c and 3d). No intermediate values for n have been observed, and the reason for the switch from $n = 2-4$ to $n = 20$ is unknown, although it appears to be related to the structural integrity of the chloroplasts. In chloroplasts lysed in 3 mM-magnesium chloride, $n = 20$ was observed only rarely. The switchover point from $n = 2-4$ to $n = 20$ varied somewhat, being sometimes as high as a/A_{max} equal to 80%, but never below 60%. All curves presented are representative of at least 20 experiments. Experiments in which the differences between duplicates was more than 2% of control for activities below 20% and more than 4% for activities above 20% were discarded. Variations in specific activity (μmol of ATP formed/h per mg of chlorophyll) of the control brought about by changes in growth conditions of the cells had no effect on the results.

Effect of TBT on ATP synthesis with NADP^+ or ferricyanide as electron acceptor. When NADP^+ or ferricyanide was used as electron acceptor, the rate of electron transport, and consequently of ATP formation, was much less than that attained in cyclic photophosphorylation with pyocyanine as cofactor. The resultant rate of ATP formation was limited by the rate of electron transport, and the kinetics of TBT inhibition duplicate those of low light-intensity (Figs. 4 and 5). Again, model curves of $n = 2-4$ provide the best agreement with the experimental points. Similar results were obtained with 2,6-dichlorophenol-indophenol as electron acceptor.

Fig. 3. Effect of TBT on ATP synthesis by chloroplasts under decreased light-intensities: (a) 1300 lx; (b) 4400 lx; (c) 11200 lx; (d) 11200 lx. The reaction mixture was as described in Fig. 2. For explanation of the theoretical curves, see the text. Numbers in parentheses represent specific activities (μmol of ATP formed/h per mg of chlorophyll) of the control, obtained under full light intensity.

Fig. 4. Effect of TBT on ATP synthesis by chloroplasts with ferricyanide as electron acceptor. The reaction mixture was as described in Fig. 2, except for the substitution of $1.5 \mu\text{mol}$ of ferricyanide for the pyocyanine. Illumination (114000 lx) was for 2 min. The number in parenthesis represents specific activity (μmol of ATP formed/h per mg of chlorophyll) of the control, obtained with pyocyanine as cofactor. For explanation of the theoretical curves, see the text.

Fig. 5. Effect of TBT on ATP synthesis by chloroplasts with NADP^+ as electron acceptor. The reaction mixture was as described in Fig. 2, except for the use of $1 \mu\text{mol}$ of NADP^+ in place of pyocyanine and the addition of saturating amounts of *Euglena* ferredoxin. Illumination (84000 lx) was for 4 min. For explanation of the theoretical curves, see the text.

DISCUSSION

The titration of chloroplasts with TBT gave a value of six ATP-synthesizing sites per electron-carrier

chain. The kinetics of inactivation at decreased electron transport show, however, a minimum value of three sites per energy-conservation chain. The two most probable explanations for this discrepancy

Scheme 1. Schematic representation of the kinetic model. (a) Shunts around energy-conservation sites (Izawa & Good, 1968); (b) non-obligatory linkage between energy-conservation chains; (c) ATP-synthesizing sites (coupling factors).

are that (1) not all TBT-binding sites represent active ATP-synthesizing sites, although they will all bind TBT equally well, or (2) there are two energy-conservation sites per electron-carrier chain, each one having three ATP-synthesizing sites (Scheme 1). The second possibility, that of two energy-conservation sites, agrees well with my experimental results in that the value of $n = 3$ remains invariant over a very wide range of activity and does not vary from one experimental condition to another. This explanation agrees with the value for $P/2e^- = 2$, which has been vigorously upheld of late (Izawa & Good, 1968; Avron & Neuman, 1968). In view of the low degree of electron-transport control by ATP synthesis observed in chloroplasts in general and in *Euglena* chloroplasts in particular, the existence of a shunt around the energy-conservation site has been suggested by Izawa & Good (1968) (a in Scheme 1). The kinetic model I suggest does not require both energy-conservation sites to be in series, as presented in Scheme 1; the sites could be on alternative electron-transport pathways such as the cyclic and non-cyclic electron-transport pathways (Avron & Neumann, 1968). All my attempts to show heterogeneity or variability in the TBT-binding sites have been negative. These attempts included varying the physiological conditions of cell growth, varying the methods of isolation of the chloroplasts and chloroplast subunits and partial purification of proteins from chloroplasts labelled with $[^{113}\text{Sn}]\text{TBT}$.

It follows clearly from the low value of n at low rates of electron transport that no intermediate pool common to a number of energy-conservation chains exists. The sudden occasional switch to a high value of n at high rates of ATP synthesis can be interpreted as a limited linkage between adjacent chains, which becomes functional only when some critical concen-

tration of an intermediate is reached. This critical concentration could thus be termed a 'spillover point', and would indicate that at least one of the components of the energy-conservation pathway is present in large molar excess over the other components (b in Scheme 1). This linkage is, however, definitely not obligatory to the overall energy-conservation reaction, the coupling of ATP synthesis to electron transport. Consequently the minimum multiple of enzyme chains in chloroplast fragments able to catalyse photophosphorylation is not limited by the energy-conservation pathway, but by some other reaction, such as oxygen evolution (Izawa & Good, 1965).

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