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STEADY-STATE KINETICS OF ADP-ARSENATE AND ATP-SYNTHESIS IN *RHODOSPIRILLUM RUBRUM* CHROMATOPHORES

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(1) Rates of ATP synthesis and ADP-arsenate synthesis catalyzed by *Rhodospirillum rubrum* chromatophores were determined with the firefly luciferase method and by a coupled enzyme assay involving hexokinase and glucose-6-phosphate dehydrogenase. (2) V_m for ADP-arsenate synthesis was about 2-times lower than V_m for ATP-synthesis. With saturating [ADP], $K(As_i)$ was about 20% higher than $K(P_i)$. With saturating [anion], K(ADP) was during arsenylation about 20% lower than during phosphorylation. (3) Plots of $1/v$ vs. $1/$[substrate] were non-linear at low concentrations of the fixed substrate. The non-linearity was such as to suggest a positive cooperativity between sites binding the variable substrate, resulting in an increased V_m/K_m ratio. High concentrations of the fixed substrate cause a similar increase in V_m/K_m, but abolish the cooperativity of the sites binding the variable substrate. (4) Low concentrations of inorganic arsenate (As_i) stimulate ATP synthesis supported by low concentrations of P_i and ADP about 2-fold. (5) At high ADP concentrations, the apparent K_i of As_i for inhibition of ATP-synthesis was 2–3-times higher than the apparent K_m of As_i for arsenylation; the apparent K_i of P_i for inhibition of ADP-arsenate synthesis was about 40% lower than the apparent K_m of P_i for ATP synthesis. (6) The results are discussed in terms of a model in which P_i and As_i compete for binding to a catalytic as well as an allosteric site. The interaction between these sites is modulated by the ADP concentration. At high ADP concentrations, interaction between these sites occurs only when they are occupied with different species of anion.

Introduction

The arsenate-uncoupling of oxidative or photosynthetic phosphorylation has been attributed to ADP-arsenate synthesis; it was hypothesized that ADP-arsenate would rapily hydrolyze, giving rise to the operation of a futile cycle [1–3]. Recently ADP-arsenate synthesis has been demonstrated in mitochondria by Gresser and co-workers [4–6], who used a coupled enzyme assay, involving hexokinase and glucose-6-phosphate dehydrogenase, as a trap for ADP-arsenate. We have extended this observation to *Rhodospirillum rubrum* chromatophores, and have introduced two other methods for the measurement of ADP-arsenate synthesis, namely, firefly luciferase and pH measurements [7–8].

Although inorganic arsenate functions as a P_i-analog for the membrane-bound ATP-synthase, surprising results have often been obtained in the past when it was used as such. For example, As_i-H_2O exchange reactions in mitochondria were insensitive to uncouplers, oligomycin and KCN, and independent of added ADP, all this in contrast with P_i-H_2O exchange [9,10]; As_i-dependent reactions in mitochondria exhibited a qualitatively different response toward the antibiotic aurovertin

Abbreviations: P_i, inorganic phosphate; BChl, Bacteriochlorophyll; $m \pm \sigma$, mean ± S.D., As_i, inorganic arsenate.

than P_i-dependent reactions [11]; and the effectiveness with which As_i competes with P_i was found to vary widely [3,9,11–14]. So now that the methods of measuring ADP-arsenate synthesis are at hand, it seems appropriate to make a detailed comparison of the kinetics of arsenylation and phosphorylation, to study their mutual inhibition, and to compare the inhibitor sensitivity of these reactions. The first two topics are the subject of this report.

Methods

R. rubrum cells were grown as described [15]. The preparation of chromatophores and the methods used to measure ADP-arsenate synthesis have been described [7]. The basal reaction mixture contained 50 mM KCl, 20 mM NaCl, 0.1 mM EDTA, 2.1 mM $MgCl_2$, 5 mM Glycylglycine, NaOH to pH 7.9, chromatophores corresponding with 13.2 μM BChl, and 0.2 μM nigericin (this was added in order to minimize postillumination phosphorylation or arsenylation). In the luciferase experiments, 25–50 μM luciferin and 1–2 μg/ml luciferase (depending on the required sensitivity) were added. The mixture (2.1 ml) was illuminated for 6 s in the case of ATP synthesis, and 15–18 s in the case of ADP-arsenate synthesis. Fig. 2 in Ref. 7 shows schematically how this method can be used to measure rates of ATP synthesis and ADP-arsenate synthesis in the same sample.

In some experiments we used the coupled enzyme assay [4] to measure ADP-arsenate or ATP synthesis. Experiments with P_i only, or with As_i only, were carried out as described previously [7]. Experiments in which both P_i and As_i were present, were carried out as follows. The basal mixture (see above) was supplemented with 6 mM glucose, 0.1 mM succinate and additions described in the text. The mixture was illuminated for 30–60 s in the presence of 30 U/ml hexokinase, in a volume of 2.8 ml. A 0.5 ml or 0.8 ml portion was assayed immediately for total product (glucose-6-phosphate and glucose-6-arsenate; this compound hydrolyzed with a half-time of 2 h at 20 °C [7]). To this end, 0.1 mM NADP and 1 U/ml glucose-6-phosphate dehydrogenase were added, and NADPH was measured by its fluorescence [7]. The remainder of the reaction mixture was shaken with 2 ml chloroform, and centrifuged in order to separate layers. 1 ml of the aqueous layer was stored overnight at −20 °C, and was then supplemented with 0.1 M HCl. This causes glucose-6-arsenate to hydrolyze rapidly. After 45 min at 0 °C the mixture was neutralized with Tris base and assayed for glucose 6-phosphate. No glucose 6-phosphate was lost in this treatment. The difference between two corresponding measurements yields the amount of glucose 6-arsenate synthesized.

During illumination, competition occurs between non-enzymic hydrolysis of ADP-arsenate, and trapping of it by hexokinase. Now hexokinase in inhibited by high concentrations of As_i [6] as well as P_i (own observations). In addition, both P_i and As_i accelerate the non-enzymic hydrolysis of ADP-arsenate [7,8]. For this reason, the P_i and As_i concentrations were not raised above 1 mM. Under those conditions, both effects were sufficiently small that when the anion concentration was varied, the proportion of ADP-arsenate (formed in the light) that was trapped by hexokinase, was approximately constant: 90% in Fig. 4, and 96% in Fig. 6. This proportion was calculated from plots such as those shown in Fig. 7 of Ref. 7.

P_i was determined colorimetrically [16]. For the determination of the P_i content of the chromatophores, 0.3 ml of a concentrated suspension (1.5–2 mM BChl) was incubated for 10 min with 3.6 ml ice-cold 6% trichloroacetic acid. After centrifugation, the P_i content of the supernatant was determined. In five batches of chromatophores the P_i content was 18 ± 6 mmol per mol BChl ($m \pm \sigma$). For the determinations of the P_i content of the arsenate solutions, 0.5 ml of the reagent was added to 2 ml aqua destillata and the absorbance change due to addition of As_i, P_i or As_i plus P_i was recorded continuously. The reagent was about as sensitive toward P_i as toward As_i. Because of the great excess of reagent, the absorbance change (measured at 720 nm) due to addition of P_i or As_i followed first-order kinetics; a plot of dE/dt vs. E_t (where E_t is the extinction at time t) was linear according to:

$$\frac{dE}{dt} = k_{ps}(E_{max} - E_t)$$

where E_{max} is the extinction after completion of the reaction. However k_{ps} (the pseudo-first-order

rate constant) was for arsenate about 130-times lower than for P_i (0.1 min^{-1} and 12.9 min^{-1}, respectively). Consequently, upon addition of a mixture of 4 nmol P_i and 800 nmol As_i, the plot of dE/dt vs. E_t was biphasic; in this case, the presence of P_i caused an increase of 50% in the initial rate of absorbance change, as compared with addition of As_i alone. These data indicated that the P_i content of the As_i-solutions was less than 0.12% (molar ratio).

BChl was estimated using an in vivo extinction coefficient of 140 $mM^{-1} \cdot cm^{-1}$ [17]. The sources of the chemicals have been given before [7].

Results

Double-reciprocal plots for the P_i-dependence of ATP synthesis were linear at high concentrations of ADP, but non-linear at low ADP concentrations (Fig. 1A). Similarly, double-reciprocal plots for the ADP-dependence of ATP-synthesis were linear at high P_i concentrations, but non-linear at low P_i concentrations (Fig. 1B). Similar results were obtained during ADP-arsenate synthesis (Fig. 2A and B). In control experiments, we obtained similar results when we used the coupled enzyme assay instead of the luciferase method (not shown). This suggests that the non-linearity was not due to accumulation of ATP or ADP-arsenate in the medium.

The (pseudo-) linear parts of the plots shown in Figs. 1B and 2B were used for slope and intercept replots. These were likewise linear (Figs. 1C and 2C) and were analyzed according to the equations:

$$\frac{V_m^x}{v^x} = 1 + \frac{K^x(\mathrm{ADP})}{[\mathrm{ADP}]} + \frac{K(\mathrm{x})}{[\mathrm{X}]}\left(1 + \frac{K'^x(\mathrm{ADP})}{[\mathrm{ADP}]}\right) \qquad (1)$$

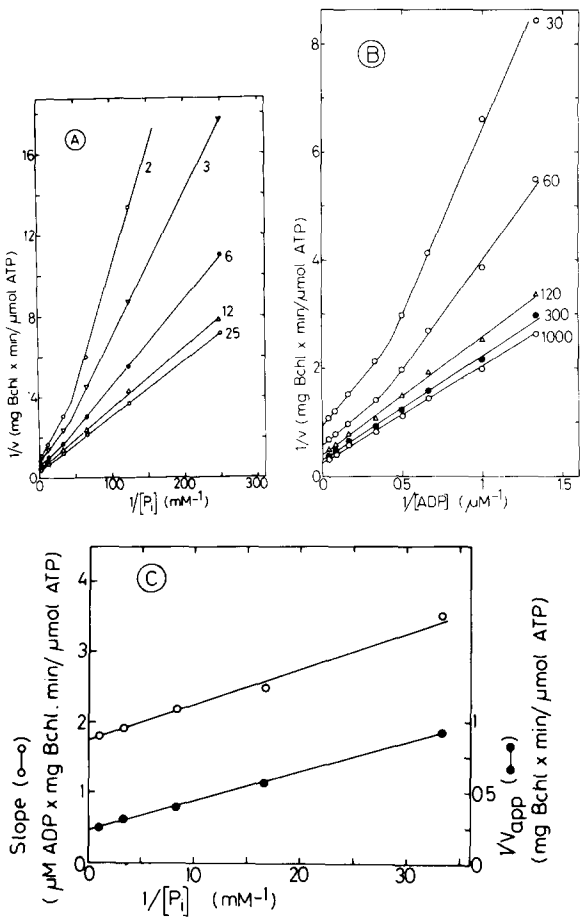


Fig. 1. Kinetics of ATP synthesis as measured with luciferase. The data (from initial rate measurements) in Fig. 1A and 1B were obtained with the same batch of chromatophores. The numbers in the figure indicate the concentrations (in µM) of ADP (panel A) and P_i (panel B). (C) Slope and intercept replot from the data shown in (B). The slopes with $[P_i]$ not over 120 µM were calculated by assuming that the primary plots (Fig. 2B) were approximately linear at ADP concentrations not below 3 µM.

TABLE I

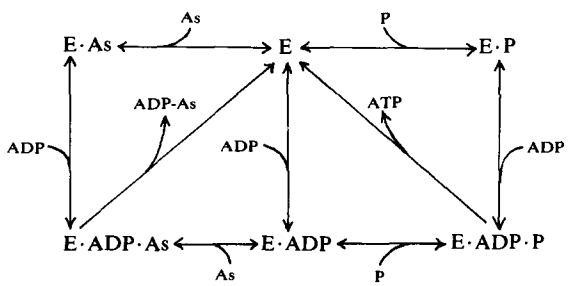
COMPARISON OF KINETIC PARAMETERS DURING ADP-ARSENATE AND ATP-SYNTHESIS

Columns 1 and 2: kinetic parameters during ADP-arsenate and ATP synthesis, respectively, as observed in five batches of chromatophores ($m \pm \sigma$). For each batch we also determined the ratio of these parameters. Column 3 shows $m \pm \sigma$ of these ratios.

	ADP-arsenate	ATP	Ratio ADP-arsenate: ATP
V_m	1.7± 0.5 µmol/min per mg Bchl	3.5± 1.0 µmol/min per mg Bchl	0.46±0.10
K_{anion}	115 ±33 µM	97 ±21 µM	1.19±0.22
K_{ADP}	6.4± 2.0 µM	7.9± 1.4 µM	0.80±0.18
K'_{anion}	152 ±54 µM	27 ± 9 µM	5.9 ±1.9
K'_{ADP}	9.2± 4.7 µM	2.2± 0.7 µM	4.7 ±3.0

$$K'(x) = K(x)\frac{K'^{x}(ADP)}{K^{x}(ADP)} \qquad (2)$$

in which X is P_i or As_i. The superscript x is introduced here to emphasize the fact that K'(ADP) and K(ADP) can have different values during arsenylation and phosphorylation. The results are summarized in Table I. Column 3 shows that V_m was during arsenylation about 2-times lower than during phosphorylation; at saturating [ADP], K_{As} was about 20% higher than K_P; and at saturating [anion], K_{ADP} was during arsenylation about 20% lower than during phosphorylation. Except for the rather low value of V_m for ADP-arsenate synthesis, these findings are in good agreement with results obtained in sub-mitochondrial particles [6]. The K_m values extrapolated to zero concentration of the second substrate (K') are shown in the bottom lines. K'_{As} was about 6-times higher than K'_P; and K'_{ADP} was during arsenylation at least 1.7-times (average: 4.7-times) higher than during phosphorylation. This cannot be explained with the simple random-addition mechanisms shown below:



If we assume that the substrates in solution are at equilibrium with the different enzyme forms, then the experimentally determined values for K'_{ADP} should be equal to the dissociation constant for the enzyme-ADP complex in the absence of anions. This will be the case, not only in the random-addition model shown above (cf. Ref. 18), but also under steady-state conditions in a simple ordered binding sequence with ADP binding first (cf. Ref. 19). Thus, both models predict that K'_{ADP} should have the same value during arsenylation and phosphorylation *.

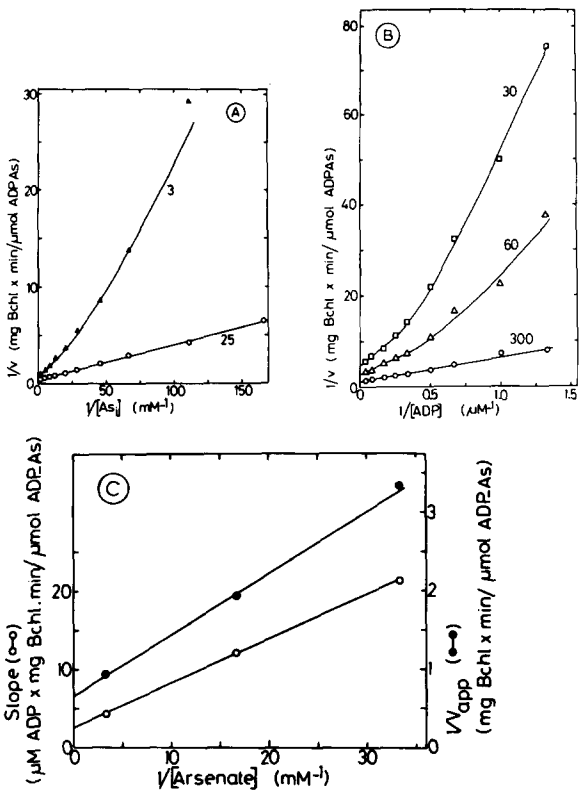


Fig. 2. Kinetics of ADP-arsenate synthesis as measured with luciferase. A value of 0.19 was assumed for the quantum yield of ADP-arsenate, relative to that of ATP, for light emission from luciferase [7]. Arsenylation rates were determined by multiplying the steady-state amount of ADP-arsenate in the light with pseudo-first order rate constant for ADP-arsenate hydrolysis [7]. The numbers in the figure indicate the concentration of ADP in µM (panel A) or of As_i in µM (panel B). The lines connecting the triangles in panel A were calculated as detailed in the Appendix. The data shown in panel B were obtained with a different batch of chromatophores from those shown in panel A. (C) Slope and intercept replot from the data shown in (B). The slopes with $[As_i] \leqslant 60$ µM were obtained by assuming that the primary plots (Fig. 2B) were approximately linear at ADP concentrations not below 3 µM.

Another prediction that can be derived from both these simple models, is that in experiments with a fixed concentration of ADP, the apparent $K_m(P_i)$ for phosphorylation should be equal to the apparent $K_i(P_i)$ for inhibition of arsenylation:

$$K_m\text{app}(P) = K_i\text{app}(P) \qquad (3)$$

* Sulphite inhibition of ATP synthesis in *Rhodospirillum rubrum* chromatophores is competitive with P_i and uncompetitive with respect to ADP. This indicates that the binding sequence of the substrates is not ordered with P_i binding first (Slooten, L., unpublished data).

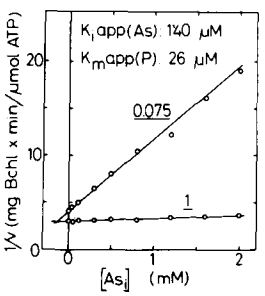


Fig. 3. Arsenate inhibition of TP synthesis supported by 2.5 μM ADP and the indicated concentrations of P_i (mM). Luciferase method; $MgCl_2$ was present at 5 mM. $K_m$app(As) (see text) was determined in the same medium but without added P_i, and with 50–1000 μM As_i.

Similarly:

$$K_m\text{app(As)} = K_i\text{app(As)} \quad (4)$$

If, in addition, the P_i concentration is held constant, the As_i-concentration required for half-saturation of ADP-arsenate synthesis ($As(S_{50})$) should be equal to the As_i concentration required for 50% inhibition of ATP synthesis ($As(I_{50})$):

$$As(S_{50}) = K_m\text{app(As)}\left(1+\frac{[P]}{K_i\text{app(P)}}\right) \quad (5)$$

and:

$$As(I_{50}) = K_i\text{app(As)}\left(1+\frac{[P]}{K_m\text{app(P)}}\right) \quad (6)$$

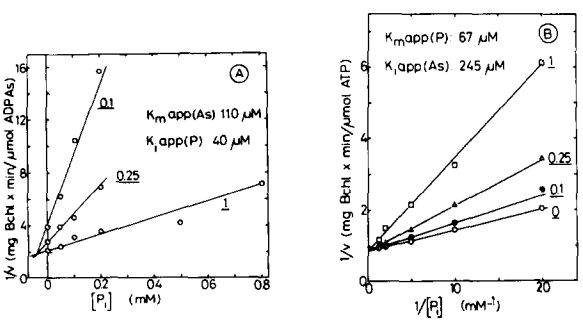


Fig. 4. Simultaneous measurement of ADP-arsenate synthesis (panel A) and ATP synthesis (panel B) with the coupled enzyme assay. The basal medium (see Methods) contained 5 mM $MgCl_2$. ADP was present at 30 μM. The numbers in the figure indicate the concentration of As_i in mM. Illumination time, 30s. In the case of ADP-arsenate synthesis, a correction of 10% was applied in order to account for non-enzymic hydrolysis of part of the ADP arsenate (see Methods).

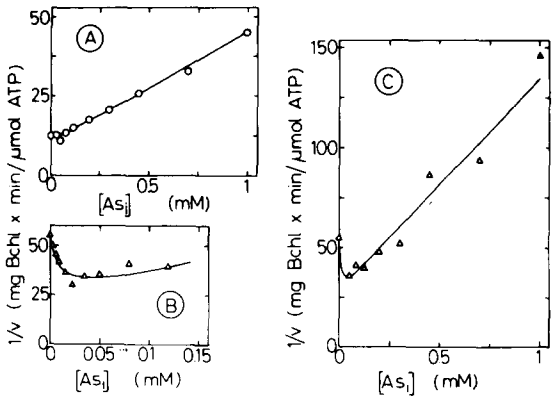


Fig. 5. Effect of As_i on ATP synthesis supported by endogenous P_i and 25 μM ADP (panel A), or 3 μM ADP (panels B and C), as measured with luciferase. These data were obtained in the same experiments as those shown in Figs. 2A and B. The lines in Fig. 5 were calculated as detailed in the Appendix.

Figs. 3–6 show experiments designed to test these predictions. In experiments with low ADP concentrations (2.5 μM) and 75 or 1000 μM P_i, $K_i$app(As) for inhibition of ATP synthesis was 140 μM (Fig. 3), in good agreement with $K_m$app(As) measured separately without added P_i: 128 μM (not shown).

Fig. 4 shows experiments in which arsenylation and phosphorylation rates were measured simultaneously (with the coupled enzyme assay), at high ADP concentrations (30 μM). Under those conditions $K_i$app(As) was 2.2-times higher than $K_m$app(As), whereas $K_i$app(P) was about 40% lower than $K_m$app(P), in contrast with Eqns. 3 and 4. Experiments done with the luciferase technique

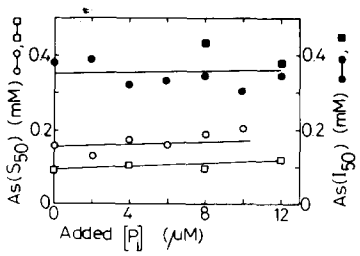


Fig. 6. Simultaneous measurement of ATP synthesis and ADP-arsenate synthesis with luciferase (○, ●) or with the coupled enzyme assay using a different batch of chromatophores (□, ■). ADP was present at 25 μM, and As_i was present at 40–800 μM. ○ and □, $As(S_{50})$; ● and ■, $As(I_{50})$. In the experiments with the coupled enzyme assay the illumination time was 1 min.

confirmed the results concerning $K_i$app(As) and $K_m$app(As) (not shown).

Fig. 5A shows the effect of As_i on ATP synthesis supported by endogenous P_i (contributed by the chromatophores; approx. 0.3 μM, see Methods), and 25 μM ADP. In that case, the inhibition kinetics (shown here in a Dixon plot) were slightly non-linear. After extrapolation of the linear part of the plot to the *Y*-axis we found a value of 330 μM for As(I_{50}). This was about 3.3-times higher than As(S_{50}) measured in the same experiments: 96 μM (Fig. 2A). Because of the low P_i concentration, As(I_{50}) and As(S_{50}) can be set equal to $K_i$app(As) and $K_m$app(As), respectively, in these experiments. Similar results were obtained when the P_i concentration was raised up to 10 or 12 μM (Fig. 6). All this is in agreement with results shown in Fig. 4, but in contrast with Eqns. 4–6. According to these equations, As(S_{50}) should in the experiments shown in Fig. 6 have been up to 15% higher than As(I_{50}); this is because $K_i$app(P) is lower than $K_m$app(P) (Fig. 4).

ATP synthesis supported by endogenous P_i and 2.5 μM ADP was stimulated by arsenatė (Figs. 5B and C). The stimulation was half-saturated at 10–15 μM As; (in different preparations). Addition of 0.2 μM P_i had about the same effect on the rate of ATP synthesis as addition of 15 μM As_i (not shown); it seemed possible, therefore, that the arsenate stimulation of ATP synthesis was due to the presence of phosphate as a contamination in the As_i stock solutions. However, this possibility was ruled out by our finding that the phosphate content of these solutions was less than 0.12% (molar ratio). Hence, the arsenate stimulation of ATP-synthesis can be attributed to interaction between an As_i-binding effector site and a P_i-binding catalytic site.

At high ADP concentrations (25 μM) the arsenate-stimulation of ATP synthesis supported by endogenous P_i amounted to only 10–15%, as indicated by the deviation from linearity of the points obtained at zero or low As_i-concentrations (Fig. 5A). The antagonistic effect of ADP on the arsenate stimulation of ATP synthesis was not due to contamination of the ADP solutions with P_i: the P_i content of the ADP stock solutions was 0.12–0.15%; however, the arsenate stimulation of ATP synthesis supported by 2.5 μM ADP was still manifest in the presence of 8 μM P_i (not shown). When, however, the P_i concentration was raised to 75 μM, the arsenate stimulation of ATP-synthesis was completely abolished (Fig. 3). This suggests competition between P_i and As_i for binding to the effector site mentioned above.

Discussion

At high ADP concentrations, the anion-dependence of both ATP synthesis and ADP arsenate synthesis exhibited linear kinetics. Yet, under these conditions $K_i$app(As) was about 2.2-times higher than $K_m$app(As), whereas $K_i$app(P) was about 40% lower than $K_m$app(P). The difference between $K_i$app(As) and $K_m$app(As) was essentially independent of the P_i concentration, down to about 0.3 μM. This indicates that, in this respect, results obtained at very low P_i concentrations cannot be dismissed as being kinetically irrelevant. It is therefore significant that when the ADP concentration was lowered to 2.5–3 μM, As_i actually stimulated ATP synthesis supported by low concentrations of P_i.

These data are indicative of site-site interactions during ATP synthesis and ADP-arsenate synthesis. The interactions might occur (A) between two identical catalytic sites, or (B) between two non-identical sites. In the latter case, we will assume the interactions to occur between an allosteric and a catalytic site. For simplicity (and also because the results concerning the discrepancies between $K_i$app(As) and $K_m$app(As) were essentially independent of the P_i concentration), we assumed equilibrium between the substrates in solution and the different enzyme forms. Under those conditions most of the data fitted with both alternatives (A) and (B). However, the data of the type shown in Fig. 5A fitted generally better with alternative (B) (see Appendix). Accordingly, we will restrict our discussion to the model shown in Fig. 7. The model assumes that interaction occurs between catalytic sites (labeled C) and allosteric sites (labeled A). It is known that the enzyme contains, in all likelihood, three catalytic sites [20–23], but these are assumed to be independent. P_i and As_i are shown to compete for both types of site. The interaction between the catalytic and allosteric site is modulated by ADP, which like-

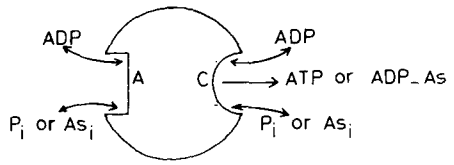


Fig. 7. Model used to account for the kinetics of ADP-arsenate and ATP synthesis in *Rhodospirillum rubrum* chromatophores. A, allosteric site; C, catalytic site. Further details, see text.

wise binds to both types of site (see below). The reaction scheme for this model is very complex, but at a given concentration of ADP it can be treated formally as a network of one-substrate reactions involving only P_i and As_i (thanks to the equilibrium assumption). With this simplification, the reaction scheme is shown in Fig. 8. (The relevant kinetic parameters are defined in the subscript.) An analysis of the data with this scheme (see Appendix) led to the following conclusions:

(1) In experiments with 25–30 μM ADP, As_i-binding at the allosteric site has no effect on the V_m/K_m ratio of ADP-arsenate synthesis (Fig. 2A), but causes a 2–3-fold increase in the V_m/K_m ratio of ATP-synthesis (Fig. 5A).

(2) In experiments with 2.5–3 μM ADP, As_i-binding at the allosteric site causes an increase in the V_m/K_m ratio of both ADP-arsenate synthesis (over 3-fold; Fig. 2A) and ATP synthesis (over 2-fold). The last-mentioned effect is manifest as an increase in the rate of ATP synthesis when $[P_i]$ is low (Fig. 5B and C).

(3) The arsenate stimulation of ATP synthesis may diminish when the ADP concentration is raised to 25–30 μM; alternatively, the As_i affinity of the allosteric site, relative to that of the catalytic site, may decrease when the ADP concentration is raised. In any case, the result is that at high ADP concentrations, the As_i-stimulation of ATP synthesis supported by endogenous P_i is expressed mainly by the fact that $K_i$app(As) is higher than $K_m$app(As) (Fig. 5A).

(4) At high ADP concentrations, P_i does bind to the allosteric site (see conclusion (6) below), but this has no effect on the V_m/K_m ratio for ATP synthesis (Fig. 1A). This is one of the reasons why $K_i$app(As) is at all P_i concentrations 2–3-times higher than $K_m$app(As) (Figs. 4–6).

(5) No such K_m-K_i discrepancy for As_i was observed in experiments with low ADP concentrations and relatively high P_i concentrations (Fig. 3). The reason is presumably that under those conditions P_i bound at the allosteric site has the same effect as As_i, i.e., it causes an increase in the V_m/K_m ratio for ATP synthesis. When the site is initially occupied with P_i, then addition of As_i will not cause a further increase in the V_m/K_m ratio. This interpretation is supported by the fact that the P_i dependence of ATP synthesis exhibits non-linear kinetics at low ADP concentrations (Fig. 1A), in a manner suggesting positive cooperativity between two P_i binding sites.

(6) P_i bound at the allosteric site causes a decrease of around 40% in the V_m/K_m ratio for ADP-arsenate synthesis supported by high ADP concentrations (Fig. 4).

(7 The non-linear primary plots in Figs. 1 and 2 suggest that a positive cooperativity between two sites binding the variable substrate occurs only when the fixed substrate is present at low concentration. Furthermore, the plots converge at increasing concentrations of the variable substrate. This may be interpreted as follows: the allosteric site may be capable of ADP binding as well as anion binding. Either ADP binding or anion bind-

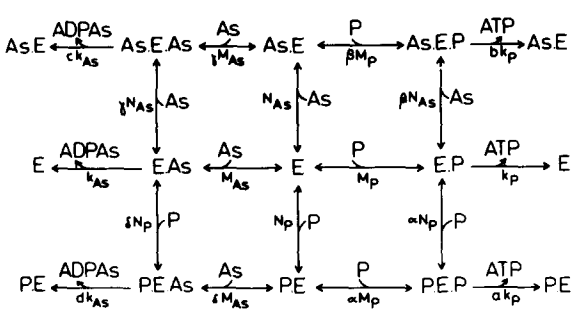


Fig. 8. A simplified (see text) reaction scheme depicting competition between P_i and As_i for binding to a catalytic and an allosteric site. The scheme shows reaction pathways as well as the kinetic constants governing these pathways. The horizontal arrows indicate reactions taking place at the catalytic site; k_{As} and k_P are apparent first-order rate constants for release of ADP-arsenate and ATP, respectively; M_{As} and M_P are apparent dissociation constants for the reaction of As_i and P_i, respectively, with the catalytic site. The vertical arrows indicate reactions taking place at the allosteric site; N_{As} and N_P are apparent dissociation constants for the reaction of As_i and P_i, respectively, with the allosteric site. The dimensionless constants $a-d$ and $\alpha-\delta$ define the strength of the interaction between the two sites. The values of all constants are determined by the ADP concentration. P, phosphate; E, enzyme.

ing to this site may cause an increase in the V_m/K_m ratio for ATP synthesis (Fig. 1) or DP-arsenate synthesis (Fig. 2). However, the effects of ADP binding and of anion binding to the allosteric site are not additive. With models based on these assumptions, one can simulate the results shown in Figs. 1B and 2B reasonably well. In addition, such models can account for the fact that K'_{ADP} was during ADP-arsenate synthesis significantly higher than during ATP synthesis (Table I).

The general features of the model shown in Figs. 7 and 8 are consistent with earlier proposals concerning the regulatory function of a substrate-binding site during ATP hydrolysis [24,25]. It is also relevant that according to recent findings tightly bound nucleotides have a regulatory rather than a catalytic function during ATP synthesis catalyzed by chloroplasts [26,27]. This is in contrast with earlier conclusions that these tightly bound nucleotides are intermediates in a flip-flop mechanism [28,29]. A flip-flop mechanism cannot explain our results, either: a steady-state treatment of such a mechanism predicts that, in experiments with very low P_i-concentrations, $As(S_{50}) = As(I_{50})$ (not shown), in contrast with the data shown in Fig. 5 and 6.

With regard to conclusion (7), it may be relevant that P_i is a partial inhibitor of tight ADP binding to the light-activated chloroplast ATPase [30–32]. It also provides a transient protection [31–33] against deactivation of the ATPase which occurs upon tight ADP binding [33]. The P_i-binding site involved in these effects is apparently different from the catalytic ATP-binding site [31]. The stimulation of ATPase activity and ATP-induced H^+-gradient formation in *Rhodopseudomonas capsulata* chromatophores by P_i and As_i [34] is presumably a related phenomenon.

Our suggestion, that the effects of P_i-binding and ADP-binding to the allosteric site are not additive during ATP synthesis, is in line with these findings. THe difference is that, in our interpretation, ADP-binding to the allosteric site stimulates ATP-synthesis (at least at low P_i concentrations). This differential effect of ADP on ATP synthesis and ATP hydrolysis might be related to the conversion of tight nucleotide-binding sites into loose-binding sites during illumination (Ref. 35; cf. Ref. 36 for chromatophores). In the experiments reported here we may have probed only the loose-binding sites.

It is not yet clear why, at high ADP concentrations, interactions between the allosteric and the catalytic site are observed only when the sites are occupied with different species of anion. However, these results may explain some of the discrepancies which in the past have turned up in experiments done with arsenate. Our finding that, with saturating [ADP], K_{As} during arsenylation is about equal to K_P during phosphorylation (Table I), confirms results obtained with submitochondrial particles [6]. However, in earlier work the K_i of As_i [3,9,11–14] was found to vary from 0.3- [13] to 10-times [11] the K_m of P_i. The reason for this large variability may be that the strength of the interaction between the catalytic and the effector site (when one of these sites is occupied with P_i and the other with As_i) varies from one organism to another.

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Appendix

The dissociation constants shown in Fig. 8 allow one to calculate the abundance of the different enzyme (E) species relative to that of the species PEP (lower right). For example, $[EP] = [PEP]\,\frac{\alpha N_P}{[P]}$, where P is phosphate; and the rate of ATP synthesis via the species EP equals $[PEP]\,\frac{\alpha N_P}{[P]}\,k_P$, where k_P is a first-order rate constant (see legend to Fig. 8). From Fig. 8 it follows that, at equilibrium, the total enzyme concentration, $[E]_t$, is given by:

$$[E]_t = [PEP]\,\alpha\left\{\frac{1}{\alpha} + \frac{M_P}{[P]}\left(1 + \frac{[As]}{\delta M_{As}}\right) + \frac{N_P}{[P]}\left(1 + \frac{[As]}{\beta N_{As}}\right) + \frac{M_P N_P}{[P]^2}\left(1 + \frac{[As]}{M_{As}} + \frac{[As]}{N_{As}} + \frac{[As]^2}{\gamma M_{As} N_{As}}\right)\right\} \qquad \text{(A-1)}$$

The rate of ATP synthesis is given by:

$$v^{P} = [PEP]k_{P}\left\{a + \frac{\alpha N_P}{[P]} + \frac{b}{\beta}\frac{\alpha N_P}{[P]}\frac{[As]}{N_{As}}\right\} \quad (A\text{-}2)$$

and the rate of ADP-arsenate synthesis is given by:

$$v^{As} = [PEP]k_{As}\frac{\alpha M_P N_P}{[P]^2}\left\{\frac{d}{\delta}\frac{[P]}{N_P}\frac{[As]}{M_{As}} + \frac{[As]}{M_{As}} + \frac{c}{\gamma}\frac{[As]^2}{M_{As}N_{As}}\right\} \quad (A\text{-}3)$$

In the absence of added P_i, the P_i concentration is sufficiently low for the rate equation for arsenylation to become approximately:

$$\frac{[E]_t k_{As} c}{v^{As}} = \frac{1 + \gamma(N_{As} + M_{As})/[As] + \gamma N_{As}M_{As}/[As]^2}{1 + (\gamma/c)N_{As}/[As]} \quad (A\text{-}4)$$

Under the same conditions, the rate equation for As_i inhibition of ATP synthesis will be:

$$\frac{[E]_t k_P}{v^P} = \frac{M_P}{[P]}\frac{1 + [As]/M_{As} + [As]/N_{As} + [As]^2/\gamma M_{As}N_{As}}{1 + (b/\beta)[As]/N_{As}} \quad (A\text{-}5)$$

In experiments with 25–30 μM ADP, the anion dependence of both ATP synthesis and ADP-arsenate synthesis exhibited linear kinetics (Figs. 1A and 2A). This suggests that under those conditions there is no interaction between the catalytic and allosteric site. Hence we may put $a = \alpha = c = \gamma = 1$, so that Eqn. A-4 reduces to:

$$\frac{[E]_t k_{As}}{v^{As}} = 1 + \frac{M_{As}}{[As]} \quad (A\text{-}6)$$

The value for M_{As} so obtained may be introduced into Eqn. (A-5). This equation can then be solved for N_{As} and b/β. (The quantity b/β represents the change in the V_m/K_m ratio for ATP synthesis due to As_i-binding at the allosteric site.) The curve in Fig. 5A was obtained using the following parameters: $M_{As} = 96$ (109 ± 41) μM; $N_{As} = 245$ (167 ± 67) μM; $N_{As}/M_{As} = 2.55$ (1.70 ± 0.88) and $b/\beta = 3.62$ (3.66 ± 0.90) (values within brackets are $m \pm \sigma$ for three batches of chromatophores). (N.B. If we use instead of Fig. 8 a model involving two identical catalytic sites, we should start from the assumption $N_{As} = M_{As}$. This yielded in most cases a poor fit to the data of the type shown in Fig. 5A.)

The data on ADP-arsenate synthesis supported by low concentrations of ADP without added P_i were analyzed with Eqn. A-4. The curves connecting the triangles in Fig. 2A were obtained using the following parameters: $(M_{As})^{-1} + (N_{As})^{-1} = 49.0$ mM^{-1}; $\gamma N_{As}M_{As} = 0.0031$ mM^2 and $(\gamma/c)N_{As} = 0.00626$ mM (hence $c/\gamma > 3.26$). The data in Fig. 5, on As_i-inhibition of ATP-synthesis supported by low ADP concentrations and endogenous P_i, were obtained in the same experiments. The curves shown in Fig. 5B and C were obtained using the following parameters: $(M_{As})^{-1} + (N_{As})^{-1} = 43.4$ mM^{-1}; $\gamma N_{As}M_{As} = 0.00464$ mM^2 and $(\beta/b)N_{As} = 0.00988$ mM (hence $b/\beta > 2.33$). The agreement between these two sets of results is close enough to be consistent with our assumption that the allosteric site involved in arsenate stimulation of ATP synthesis is also responsible for the non-linear kinetics of ADP-arsenate synthesis at low ADP concentrations (Fig. 2A).

At low As_i-concentrations, the quadratic term in Eqn. A-5 can be neglected. Hence low concentrations of arsenate will stimulate ATP-synthesis supported by endogenous P_i only if $b/\beta > 1 + N_{As}/M_{As}$. This is the case at low ADP concentrations but not at high ADP concentrations. This suggests that an increase in the ADP concentration causes either a decrease in b/β, or an increase in N_{As}/M_{As}.

Since it is not possible to measure arsenylation with low As_i-, and high P_i concentrations, we have no direct evidence for the magnitude of N_P/M_P or d/δ. (The quantity d/δ represents the change in the V_m/K_m ratio for ADP arsenate synthesis due to P_i-binding at the allosteric site.) However, a crude estimate can be made as follows. In the experiments shown in Fig. 4, both P_i inhibition of ADP-arsenate synthesis and As_i inhibition of ATP synthesis exhibited, to a good approximation, linear kinetics, indicative of competitive inhibition. Because of the high ADP concentration, we may again put $a = \alpha = c = \gamma = 1$. Under those conditions, it can be shown from Eqns. A-1–A-3 that pure competitive inhibition will be obtained only when the following conditions are met: $b/\beta = N_{As}/M_{As}$, $d/\delta = N_P/M_P$ and $\beta\delta = b + d - bd$. Under those conditions, $K_i\text{app(As)} = N_{As}$ and $K_i\text{app(P)} = N_P$. Furthermore, $K_m\text{app(As)} = M_{As}$

(Eqn. A-6), and similarly, $K_m app(P) = M_P$. Thus, the data in Fig. 4 indicate that $N_{As}/M_{As} \approx 2.2$ ($\pm 50\%$), and $b/\beta \approx 2.2$ ($\pm 30\%$); similarly, $N_P/M_P \approx 0.6$ ($\pm 50\%$) and $d/\delta \approx 0.6$ ($\pm 30\%$). The range added in brackets was estimated on the basis of model simulations. The results for b/β and N_{As}/M_{As} obtained from Fig. 4 are not inconsistent with those mentioned in connection with Fig. 5A.

References

1 Ernster, L., Lee, C.-P. and Janda, S. (1967) in Biochemistry of Mitochondria (Slater, E.C., Kaninga, Z. and Wojtzak, L., eds.), pp. 29–51, Academic Press, New York

2 Bertagnolly, B.L. and Hanson, J.B. (1973) Plant Physiol. 52, 431–435

3 Avron, M. and Jagendorf, A.T. (1959) J. Biol. Chem. 234, 967–972

4 Gresser, M.J. (1981) J. Biol. Chem. 256, 5981–5983

5 Moore, S.A. and Gresser, M.J. (1982) Fed. Proc. 41, 749

6 Moore, S.A., Moennich, D.M.C. and Gresser, M.J. (1983) J. Biol. Chem. 258, 6266–6271

7 Slooten, L. and Nuyten, A. (1983) Biochim. Biophys. Acta 725, 49–59

8 Slooten, L. and Nuyten, A. (1984) Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. II, pp. 615–618, Martinus Nijhoff/Dr. W. Junk Publishers, The Hague

9 Mitchell, R.A., Chang, B.F., Huang, C.H. and DeMaster, E.G. (1971) Biochemistry 10, 2049–2054

10 DeMaster, E.G. and Mitchell, R.A. (1970) Biochem. Biophys. Res. Commun. 39, 199–203

11 Ter Welle, H.F. and Slater, E.C. (1967) Biochim. Biophys. Acta 143, 1–17

12 Melandri, B.A., Baccarini-Melandri, A., San Pietro, A. and Gest, H. (1970) Proc. Natl. Acad. Sci. USA 67, 477–484

13 Lehninger, A.L. and Gregg, C.T. (1963) Biochim. Biophys. Acta 78, 12–26

14 Horio, T. and Yamashita, J. (1964) Biochim. Biophys. Acta 88, 237–250

15 Slooten, L. and Branders, C. (1979) Biochim. Biophys. Acta 547, 79–90

16 Tausski, H. and Shorr, E. (1953) J. Biol. Chem. 202, 675–685

17 Clayton, R.K. (1963) in Bacterial Photosynthesis (Gest, H., San Pietro, A. and Vernon, L.P., eds.), pp. 495–500, Antioch Press, Yellow Springs, OH

18 Kayalar, C., Rosing, J. and Boyer, P.D. (1977) J. Biol. Chem. 252, 2486–2491

19 Selman, B.R. and Selman-Reimer, S. (1981) J. Biol. Chem. 256, 1722–1726

20 Cross, R.L. (1981) Annu. Rev. Biochem. 50, 681–715

21 Satre, M., Bof, M., Issartel, J.P. and Vignais, P.V. (1982) Biochemistry 21, 4772–4776

22 Issartel, J.P., Klein, G., Satre, M. and Vignais, P.V. (1983) Biochemistry 22, 3485–3491

23 Issartel, J.P., Klein, G., Satre, M. and Vignais, P.V. (1983) Biochemistry 22, 3492–3496

24 Schuster, S.M., Ebel, R.E. and Lardy, H.A. (1975) J. Biol. Chem. 250, 7848–7853

25 Recktenwald, D. and Hess, B. (1977) FEBS Lett. 76, 25–28

26 Aflalo, C. and Shavit, N. (1982) Eur. J. Biochem. 126, 61–68

27 Tiefert, M.A. and Shavit, N. (1983) J. Bioenerg. Biomembrane 15, 257–276

28 Boyer, P.D. and Kohlbrenner, W.E. (1981) in Energy coupling in Photosynthesis (Selman, B.A. and Selman-Reimer, S., eds.), pp. 231–240, Elsevier/North-Holland, Amsterdam

29 Smith, D.J. and Boyer, P.D. (1976) Proc. Natl. Acad. Sci. USA 73, 4314–4318

30 Schumann, J. and Strotmann, H. (1980) Proceedings of the 5th International Congress on Photosynthesis Research (Akoyunoglou, G., ed), Vol. II, 881–892, Balaban International Science Services, Philadelphia, PA

31 Schumann, J. (1981) in Energy Coupling in Photosynthesis (Selman, B.R. and Selman-Reimer, S., eds.), pp. 223–230, Elsevier/North-Holland, Amsterdam

32 Dunham, K.R. and Selman, B.R. (1981) J. Biol. Chem. 256, 10044–10049

33 Carmeli, C. and Lifshitz, Y. (1972) Biochim. Biophys. Acta 267, 86–95

34 Baccarini-Melandri, A., Fabbri, E. and Melandri, B.A. (1975) Biochim. Biophys. Acta 376, 82–88

35 Strotmann, H., Bickel-Sandköttner, S., Franek, U. and Gerke, V. (1981) in Energy Coupling in Photosynthesis (Selman, B.R. and Selman-Reimer, S., eds.) pp. 187–196, Elsevier/North-Holland, Amsterdam

36 Slooten, L. and Nuyten, A. (1981) Biochim. Biophys. Acta 638, 313–326