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Gregory A. Grant

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Elucidation of a Self-Sustaining Cycle in *E. coli* L-Serine Biosynthesis that Results in the Conservation of the Coenzyme, NAD^+.

Gregory A. Grant

From the Departments of Developmental Biology and Medicine, Washington University School of Medicine, 660 S. Euclid Avenue, Box 8103, St. Louis, Missouri 63110.

Running Title: Conservation of NAD^+ in *E.coli* Serine Biosynthesis

To whom correspondence should be addressed: Prof. Gregory A. Grant, Department of Developmental Biology, Box 8103, Washington University School of Medicine, St. Louis, MO 63110. Phone 314-362-3367, FAX 314-362-4698, email ggrant@wustl.edu.

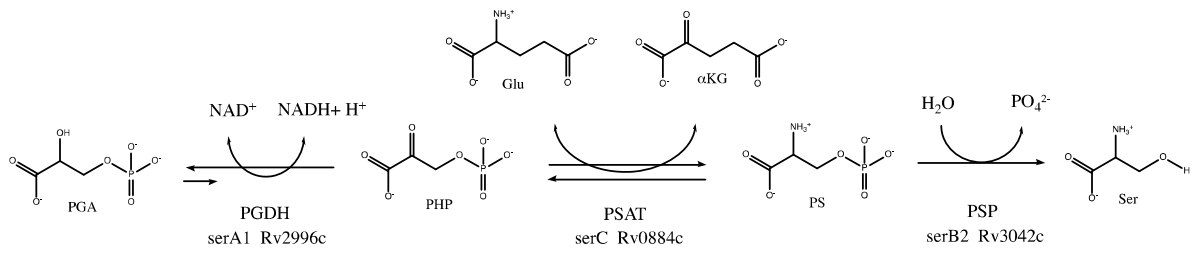
Keywords:

ABSTRACT

The equilibrium of the reaction catalyzed by D-3-Phosphoglycerate Dehydrogenase (PGDH), the first enzyme in the L-serine biosynthetic pathway, is far in the direction away from serine synthesis. As such, the enzyme is usually assayed in this direction. In order to easily assay it in the direction of L-serine synthesis, it can be coupled to the next enzyme in the pathway, phosphoserine aminotransferase (PSAT), with the activity monitored by the conversion of NAD^+ to NADH by PGDH. However, when PGDH from several different species were coupled to PSAT, it was found that one of them, *ec*PGDH, conserves coenzyme in the production of L-serine by utilizing an intrinsic cycle of NAD^+/NADH interconversion coupled with the conversion of α-ketoglutarate (αKG) to α-hydroxyglutarate (αHG). Furthermore, the cycle can be maintained by production of αKG by the second enzyme in the pathway, PSAT, and does not require any additional enzymes. This is not the case for PGDH from another bacterial source, *M. tuberculosis*, and a mammalian source, human liver, where net consumption of NAD^+ occurs. Both NAD^+ and NADH appear to remain tightly bound to *ec*PGDH during the cycle, effectively removing a requirement for the presence of an exogenous coenzyme pool to maintain the pathway and significantly reducing the energy requirement needed to maintain this major metabolic pathway.

Introduction

Nearly all organisms, from bacteria to mammals, contain a pathway for the production of L-serine from the glycolytic intermediate, D-3-phosphoglycerate (PGA, 1-9). D-3-Phosphoglycerate dehydrogenase (PGDH) initiates the entry of PGA into the pathway (2, 3, 5-9) by converting it to phosphohydroxypyruvate (PHP) with the concomitant reduction of NAD^+ (Scheme 1). PHP is then converted to phosphoserine (PS) along with the conversion of glutamate (Glu) to α-ketoglutarate (αKG) by phosphoserine aminotransferase (PSAT). Lastly, phosphoserine is converted to L-serine and phosphate in an irreversible reaction by phosphoserine phosphatase (PSP).



Scheme 1. The L-Serine Biosynthetic Pathway. PGA, D-3-phosphoglycerate; PHP, phosphohydroxypyruvate; PS, phosphoserine; Ser, L-serine; NAD^+/NADH, nicotinamde adenine dinucleotide; Glu, L -glutamic acid; αKG, alpha-ketoglutarate; PGDH, D-3-phospoglycerate dehydrogenase; PSAT, phosphoserine aminotransferase; PSP, phosphoserine phosphatase.

L-Serine occupies a pivotal position in carbon metabolism due to its role as a precursor for a wide array essential metabolites (8-9). In addition to incorporation into proteins, L-serine is central to the production of many other metabolites such as glycine, cysteine, tryptophan, phosphatidyl L-serine, sphingolipids, purines, porphyrins and glyoxalate. As the major precursor to glycine, it also contributes the one-carbon unit (C1) that is the donor in methylation reactions mediated by derivatives of tetrahydrofolate and S-adenosyl methionine.

The genes encoding PGDH, PSAT, and PSP in Proteobacteria are designated *serA1*, *serC*, and *serB2*, respectively. In *Mycobacteria tuberculosis* (*Mtb*) they are designated Rv2996c, Rv0884c, and Rv3042c, respectively.

Many bacteria also contain genes designated *serA2* and *serB1in* Proteobacteria *and Rv0728c and Rv0505c in Mtb*. However, only *serA1* and *serB2* have been shown to be essential (10, 11). *SerA2* is a D-glycerate dehydrogenase (also called hydroxypyruvate reductase or glyoxylate dehydrogenase) that is often claimed to be a second, non-phosphorylated pathway for serine biosynthesis that interconverts hydroxypyruvate and D-glycerate with the concomitant conversion of NADH and NAD^+. However, as pointed out by Snell (12) for mammalian systems, this is a misconception. This enzyme is located in the mitochondria in mammals (13) and in the peroxisome in plants (9) and functions in a catabolic role in a pathway that converts L-serine to D-2-phosphoglycerate in which hydroxypyruvate and D-glycerate are intermediates. The *serA2* gene in *E. coli* is also a hydroxypyruvate reductase (Figure S1) and is also not likely to be involved in *de novo* serine biosynthesis in *E. coli*. This is supported by the observation that when *serA1* is knocked out, the bacteria require exogenous serine for growth (14). The "phosphorylated" pathway, utilizing *serA1*, is therefore the only anabolic source of L-serine in most organisms. In *Escherichia coli*, for example, approximately 15% of the carbon assimilated when it is grown on glucose passes through L-serine before incorporation into biosynthetic products (15). *serB1* has proven difficult to express and isolate and has not been studied further (16, 17), but as mentioned earlier, it is not essential for growth.

Interestingly*, E. coli* PGDH (*ec*PGDH) can utilize αKG as a substrate in the reverse direction (the direction opposite that toward serine synthesis), converting it to α-hydroxyglutarate (αHG) with the conversion of NADH to NAD^+ (14). In addition to *ec*PGDH, the ability to use αKG as a substrate has also been reported for *Pseudomonas stutzeri* (*ps*PGDH) (19), *Pseudomonas aeruginosa* (*pa*PGDH) (19), *S. cerevisiae* (*sc*PGDH) (20), and human PGDH (*hs*PGDH) (21). However, this is apparently not the case for other PGDHs such as that from *Mycobacterium tuberculosis* (*mt*PGDH) (22) and *Rattus norvegicus* (23).

NADH binds very tightly to *ec*PGDH and *ps*PGDH. For *ec*PGDH, the K_d of binding has been estimated to be less than 10 nM and NADH binding induces a conformational change that essentially

locks the coenzyme in the protein (18). The K_d of binding for *ps*PGDH is reported to be ~ 0.1 μM by isothermal titration calorimetry (19). The binding of substrate and coenzyme is ordered, but the order differs among species. The order of binding for *ec*PGDH (34, 35) and *ps*PGDH (19) is coenzyme before substrate, while that for *mt*PGDH is substrate before coenzyme (36).

Consistent with this, *ec*PGDH (34, 37) and *ps*PGDH (19) contain at least 2 mols of very tightly bound NADH per mol of enzyme tetramer, as measured by the fluorescence of NADH, when they are isolated. The NADH bound to isolated *ec*PGDH cannot be removed by dialysis (34). Furthermore, the bound NADH cannot be displaced by the addition of NAD^+. Since NADH is produced *in situ* with each turnover of the enzyme, this would seem to present a problem for the cell since NAD^+ must bind to the enzyme prior to substrate binding for the production of L-serine to proceed. Therefore, since *E. coli* efficiently produces L-serine *in vivo*, some compensating mechanism must be operational. In a recent review (38), Gusyatiner and Ziyatdinov hypothesized that αHG production from αKG by *ec*PGDH is necessary to convert the bound NADH to NAD^+ in order for the forward reaction to proceed toward L-serine biosynthesis. However, this review presented the hypothesis only and no experimental results confirming it were included. In addition, a recent report by Zhang et. al. suggested that coupling between *ps*PGDH and a D-2-hydroxyglutarate dehydrogenase (D2HGDH) drives L-serine production (19). Although they demonstrated that *ps*PGDH was capable of converting αKG to αHG, they concluded that robust coupling between *ps*PGDH and D2HGDH drives L-serine production in *Pseudomonas*. Furthermore, since they were unable to find a D2HGDH homolog in *E. coli*, they concluded that other unknown enzymes were involved in D-2-HG catabolism in *E. coli*.

The investigation reported here presents experimental *in vitro* evidence that demonstrates that αKG is not required to initiate L-serine biosynthesis in *E. coli* and that a D2HGDH counterpart is also not required for efficient recycling of αKG or the synthesis of L-serine. On the contrary, *ec*PGDH utilizes an internal cycle that preserves NAD^+ by constantly interconverting the NADH produced back to NAD^+ through a reaction with αKG that is produced by the next coupled enzyme in the pathway, PSAT. This

investigation also demonstrates that in addition to tightly bound NADH, *ec*PGDH also contains tightly bound NAD^+ so that exogenous NAD^+ is not required for the cycle to be functional *in vitro*. This work also demonstrates that this is not the case for *tb*PGDH or *hs*PGDH, and that *hs*PGDH does not efficiently utilize αKG as a substrate to produce αHG.

EXPERIMENTAL PROCEDURES

PGDH, PSAT, and PSP from *E. coli* and *M. tuberculosis* are produced with hexa-histidine tags, expressed in *Escherichia coli* BL21 DE3 cells, and purified as previously described (39). D-glycerate dehydrogenase from *E. coli* (*serA2*) was cloned from *E.coli* DNA by PCR amplification as previously described for *tb*PGDH (36). All reagents were purchased from Sigma Aldrich, Inc. The assays presented in Table 1 were run in duplicate in 100 mM KPO_4 buffer, pH 7.0, initiated with the addition of PGA, and monitored at 340 nm for the production of NADH. Assays contained, 2.5 mM PGA, 1.25 mM NAD^+, 200 mM Glu, and 6.8 mM αKG when present. Enzyme concentrations were 3.4 μM *ec*PGDH, 0.4 μM *tb*PGDH, 1.8 μM *hs*PGDH, and 0.5 μM PSAT. The colorimetric phosphate assays were performed by a modification of the assay of Chen et.al. (40) as reported on-line by Dunham (Dunham.gs.washington.edu/MDphosphateassay.htm). NADH bound to *ec*PGDH was converted to NAD^+ by reaction with αKG followed by washing with 10 volumes of 50 mM MOPS buffer, pH 7.0 using an Amicon Ultra centrifugal filter with 10 kDa molecular weight cutoff. NAD^+ bound to *ec*PGDH was converted to NADH by reaction with PGA after washing with 10 volumes of 50 mM MOPS buffer, pH 7.0 using an Amicon Ultra centrifugal filter with 10 kDa molecular weight cutoff. Fluorescence due to NADH was measured by scanning from ~ 370-490 nm with excitation at 340 nm. The assays presented in Table 2 were run in duplicate in 50 mM MOPS buffer pH 7.0, 5 mM $MgCl_2$. When present, PSP was 1 μM and all other reagent and enzyme concentrations were as listed for Table 1. Reactions in Table 2 were initiated with the addition of PGA and allowed to run for 10 min. Then PSP was added and allowed to incubate for 1 min before addition of phosphate assay reagent which stopped the reaction. The data in

Figure 2 was produced in the same manner except the reaction was allowed to proceed for various times prior to the addition of PSP. The data in Figure 5 was produced with PGDH, PSAT, and PSP present at the beginning and allowed to run for 10 minutes before adding assay reagent to stop the reaction. Color development proceeded for 1.5 h at 37ºC and the absorbance at 820 nm was recorded. Phosphate concentration was determined by comparison to a standard curve. Absorbance due to NADH was monitored at 340 nm in separate experiments.

Kinetic parameters for PGDH in the forward reaction direction were determined by coupling PGDH and PSAT and monitoring the production of NADH at 340 nm. The coupled reaction was optimized so that the initial enzyme, PGDH, was rate limiting and the rate was determined with the linear portion of the curve where the intermediate, PHP, reaches a steady state following an initial lag phase. Plots of activity versus substrate concentration were fit to the Michaelis-Menten equation for a cooperative process,

$$v = V_m[S]^n / K_m^n + [S]^n \qquad \text{(Eq. 1)}$$

where v is the velocity at substrate concentration S, V_m is the maximum velocity, K_m is the substrate concentration at half-maximal velocity, and n is the Hill coefficient.

RESULTS

E. coli PGDH does not show net production of NADH in the forward direction when coupled with PSAT. The equilibrium of the reaction of PGDH interconverting PGA/NAD^+ and PHP/NADH lies far in the direction of PGA/NAD^+ production (19, 41). It is very difficult to assay the enzyme in the forward direction, and historically, fluorescence monitoring was necessary for sensitivity and hydrazine was added, presumably to trap the PHP produced (41). At 25º C, the ratio of the rates of the reverse to the forward reaction was reported to be 70 for *ec*PGDH (41). Because of this, catalytic activity assays are most often conducted in the reverse reaction direction by measuring the disappearance of NADH. However, theoretically, if the PGDH forward reaction is coupled to the second enzyme in the pathway, PSAT (Scheme 1), a facile forward reaction for PGDH should be able to be observed by monitoring the

absorbance of NADH produced. Coupled reactions between PGDH and PSAT have been utilized for *hs*PGDH (42, 43) in screening chemical libraries for inhibitors and for *ps*PGDH (19). However, neither study reported the kinetic parameters. In order to better quantify the parameters of the forward coupled reaction, this study investigated the forward kinetic parameters of PGDH from *E. coli*, *M. tuberculosis*, and human liver. The first efficiently utilizes αKG as a substrate, the second does not, and the third has been reported to use it (21), but only inefficiently. The results are shown in Table 1.

Table 1
Kinetic Parameters of PGDH Forward Reaction Coupled with PSAT[a]

Enzyme Variable Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat} / K_m ($M^{-1}s^{-1}$)	n_H
*tb*PGDH				
PGA	0.54 ± 0.08	1.3 ± 0.1	2.4 x 10^3	0.8 ± 0.1
NAD	0.06 ± 0.01	1.5 ± 0.1	2.5 x 10^4	1.2 ± 0.1
*hs*PGDH				
PGA	0.48 ± 0.10	0.38 ± 0.02	0.8 x 10^3	0.63 ± 0.06
NAD	0.49 ± 0.14	0.42 ± 0.03	0.9 x 10^3	0.67 ± 0.10
*ec*PGDH				
PGA	NATBD[b]			
NAD	NATBD[b]			

[a] 50 mM MOPS pH 7.0.
[b] NATBD, not able to be determined by monitoring A_{340}.

The forward reaction of *mt*PGDH and *hs*PGDH could be easily monitored by following the production of NADH using its absorbance at 340 nm (Figure 1, Table 1, and Table 2, reaction 1, 2 and 3). The kinetic parameters for the two enzymes are similar, however, *hs*PGDH displays a higher requirement for NAD^+ and marked negative cooperativity. On the other hand, the forward reaction assay for *ec*PGDH showed no change in absorbance at 340 nm (Figure 1 and Table 2, reactions 4 and 5) even though the reverse reaction using either αKG or PHP showed robust conversion of NADH to NAD^+ (not shown), indicating a very active enzyme preparation. The same thing is observed using *ec*PGDH that has been

depleted of NADH (Table 2, reaction 6). Interestingly, when *tb*PGDH is added to the reaction mixture containing *ec*PGDH, a robust increase in absorbance at 340 nm is observed (Figure 1, and Table 2, reaction 7). Furthermore, the coupled reactions are not specific for the origin of the PSAT as *ec*PSAT and *tb*PSAT yield similar results (not shown). It is particularly interesting to note that when *ec*PGDH is added to the reaction mixture with *tb*PGDH (reaction 7), the rate of NADH production appears to be reduced from that with *tb*PGDH alone (reactions 1 and 2), and it is completely eliminated with the addition of αKG (reaction 8). This is likely due to the ability of *ec*PGDH to use αKG to convert NADH back to NAD^+.

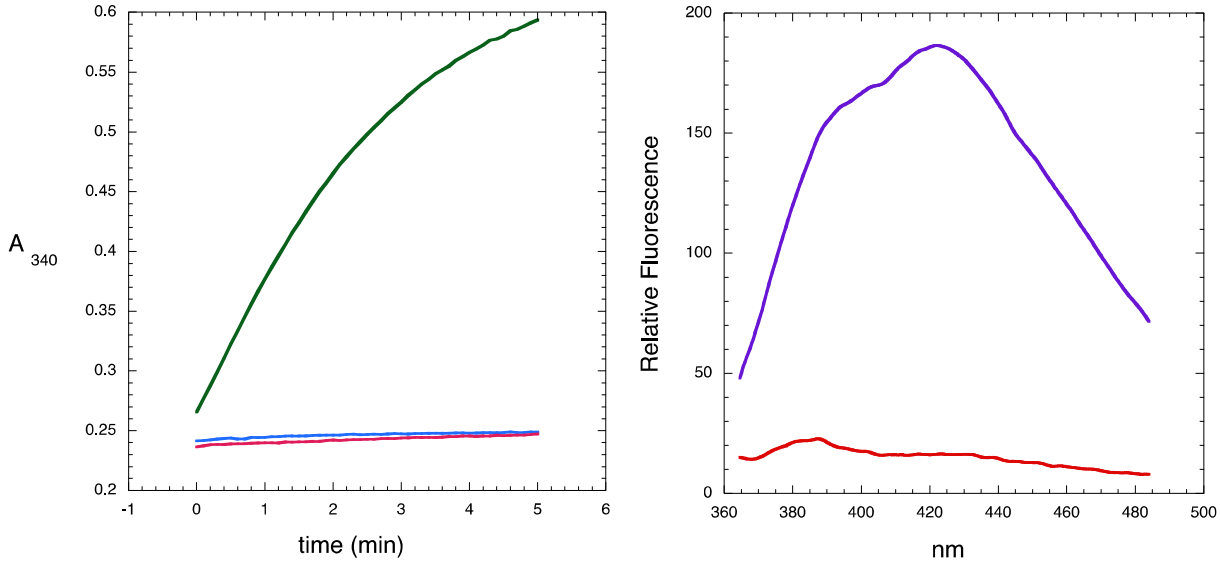


Figure 1. Left: Generation of NADH monitored by absorbance at 340 nm. Blue, reaction contains PGA, NAD^+, and either *ec*PGDH or $ecPGDH_{apo}$; Red, reaction contains PGA, NAD^+, *ec*PGDH, PSAT, and Glu; Green, reaction contains PGA, NAD^+, *ec*PGDH, PSAT, Glu, and *tb*PGDH. Abbreviations are the same as used in the text. **Right:** Fluorescence emission spectra of *ec*PGDH excited at 340 nm with bound NADH (blue) and after NADH has been oxidized by the addition of αKG (red).

Table 2
Catalytic Activity of PGDH Reaction Mixtures

Rx#	Reagents[a]				Enzymes[a]			μM/min[b]
1.	PGA	NAD^+	Glu		PSAT	*tb*PGDH		38±1
2.	PGA	NAD^+	Glu	αKG	PSAT	*tb*PGDH		34±1
3.	PGA	NAD^+	Glu		PSAT	*hs*PGDH		45±1
4.	PGA	NAD^+	Glu		PSAT		*ec*PGDH	<0.1
5.	PGA	NAD^+	Glu	αKG	PSAT		*ec*PGDH	<0.1
6.	PGA	NAD^+	Glu		PSAT		$ecPGDH_{apo}$	<0.1
7.	PGA	NAD^+	Glu		PSAT	*tb*PGDH	*ec*PGDH	22±1
8.	PGA	NAD^+	Glu	αKG	PSAT	*tb*PGDH	*ec*PGDH	<0.1

[a] PGA, D-3-phosphoglycerate; NAD^+, oxidized nicotinamide adenine dinucleotide; Glu, glutamic acid; αKG, α-ketoglutarate; PSAT, phosphoserine aminotransferase; *tb*PGDH, D-3-phosphoglycerate dehydrogenase from *M. tuberculosis*; *ec*PGDH, D-3-phosphoglycerate dehydrogenase from *E. coli*; $ecPGDH_{apo}$, D-3-phosphoglycerate dehydrogenase from *E. coli* that has been enzymatically depleted of NADH. *hs*PGDH, D-3-phosphoglycerate dehydrogenase from human liver.
[c] Generation of NADH measured by an increase in absorbance at 340 nm.

Production of phosphoserine in the forward reaction direction. Based on the above observations, one can hypothesize that when *ec*PGDH is present, net production of NADH is not observed because it is efficiently converted back to NAD^+ in the presence of αKG. The reduced production of NADH in reaction 7 is likely due to *ec*PGDH using the relatively low levels of αKG produced by the PSAT reaction to convert some, but not all, of the NADH produced back to NAD^+. The net production of NADH is eliminated if excess αKG is supplied (reaction 8). If this conclusion is correct, even if no net production of NADH is observed spectrophotometrically, phosphoserine should be produced along with αKG in the PSAT reaction (Scheme 1). To test this, phosphoserine phosphatase (PSP) is added to various reaction mixtures after an initial period of incubation and the amount of phosphate produced as a consequence of phosphoserine conversion to serine (Scheme 1) is measured colorimetrically. The net production of NADH is monitored by change in absorbance at 340 nm (Table 3).

Table 3
Phosphoserine and Net NADH Production by Reaction Mixtures

	Substrates[a]				Enzymes[a]			$[PO_4]$ μM[b]	μM/min[c]
1.	**PGA**	**NAD^+**	**Glu**		**PSAT**	**ecPGDH**	**PSP**	**30.2 ± 2.4**	**<0.1**
2.	**PGA**	**NAD^+**	**Glu**	**αKG**	**PSAT**	**ecPGDH**	**PSP**	**62.8 ± 1.6**	**<0.1**
3.	**PGA**		**Glu**		**PSAT**	**ecPGDH**	**PSP**	**23.3 ± 0.3**	**<0.1**
4.	**PGA**	**NAD^+**	**Glu**		**PSAT**	**tbPGDH**	**PSP**	**90.6 ± 4.2**	**65.6 ± 1.8**
5.	**PGA**	**NAD^+**	**Glu**	**αKG**	**PSAT**	**tbPGDH ecPGDH**	**PSP**	**111.0 ± 2.1**	**<0.1**
6.	PGA						PSP	0.25 ± 0.06	
7.	PGA	NAD^+	Glu	αKG	PSAT		PSP	1.0 ± 0.1	<0.1
8.	PGA	NAD^+	Glu	αKG	PSAT	ecPGDH		0.91 ± 0.02	<0.1
9.	PGA	NAD^+	Glu	αKG		ecPGDH	PSP	1.9 ± 0.3	<0.1

[a] PGA, D-3-phosphoglycerate; NAD^+, oxidized nicotinamide adenine dinucleotide; Glu, glutamic acid; αKG, α-ketoglutarate; PSAT, phosphoserine aminotransferase; *tb*PGDH, D-3-phosphoglycerate dehydrogenase from *M. tuberculosis*; *ec*PGDH, D-3-phosphoglycerate dehydrogenase from *E. coli*; PSP, phosphoserine Phosphatase.
[b] Phosphate determined colorimetrically.
[c] Generation of NADH measured by an increase in absorbance at 340 nm.

In Table 3, the principal reactions are in bold print with the other reactions serving as controls. Reaction 1 shows that even without added αKG, the reaction with *ec*PGDH produces significant levels of phosphoserine but net production of NADH is not observed spectrophotometrically. Reaction 2 shows similar results when αKG is added and shows that the level of phosphoserine produced increases. Remarkably, reaction 3 shows that significant phosphoserine is produced even in the absence of added NAD^+ and αKG. Furthermore, the level of phosphoserine produced is far more than the level of intrinsically bound NADH on the enzyme. Reaction 4 shows that when *tb*PGDH is used instead of *ec*PGDH, both phosphoserine and net NADH production is observed. However, reaction 5 shows that when *ec*PGDH is included along with *tb*PGDH in the presence of added αKG, phosphoserine production is essentially the same but no net production of NADH is seen as was the case with reaction 8 in Table 1.

Reaction 6 demonstrates that PGA is not a substrate for PSP. Reactions 7-9 show that *ec*PGDH, PSAT, and PSP are required for phosphate production.

ecPGDH also contains intrinsically bound NAD$^+$. The unexpected observation that phosphoserine is produced even in the absence of added NAD^+ and αKG was explored further by monitoring the reaction with respect to time. Figure 2 shows that phosphoserine accumulates as a function of time and eventually reaches a plateau. Therefore, the color produced cannot be attributed to the presence of phosphate in the initial reaction mixture. Since NAD^+ is required in the net conversion of PGA to PSP, the only logical explanation for this observation is that *ec*PGDH contains intrinsically bound NAD^+.

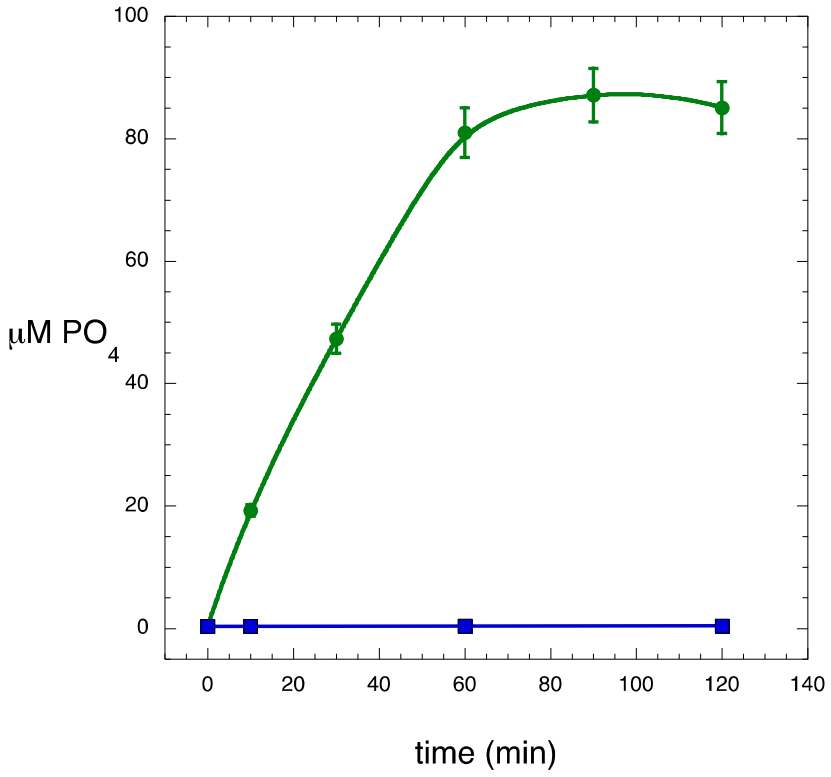


Figure 2. Time course of phosphate generation by *ec*PGDH in the absence of added NAD^+ and αKG. Reaction mixtures contained PGA, glutamate, *ec*PGDH, PSAT, with (green circles) or without (blue squares) PSP. The reaction is started by the addition of PGA and allowed to proceed. At the designated times, aliquots are removed and added to the phosphate colorimetric reagent to stop the reaction and develop color. Data is the average of duplicate determinations and error bars represent ± SEM. If error bars are not evident, they are within the area of the symbol.

To test this, the intrinsically bound NADH in a fresh isolate of *ec*PGDH was converted to NAD^+ by the addition of αKG. When conversion was complete as monitored by fluorescence analysis, the enzyme was extensively washed by repeated cycles of buffer exchange using an Amicon Ultracentrifugal filter with 10 kDa molecular weight cutoff. After washing, the fluorescence of the resulting protein showed that it did not contain any appreciable amounts of NADH (Figure 3). However, upon addition of PGA to the sample, a very strong fluorescence signal was generated indicating the production of NADH. Therefore, since NAD^+ was not added to the reaction but is required for the generation of NADH coupled to PGA conversion to PHP, it must have been bound to the enzyme initially.

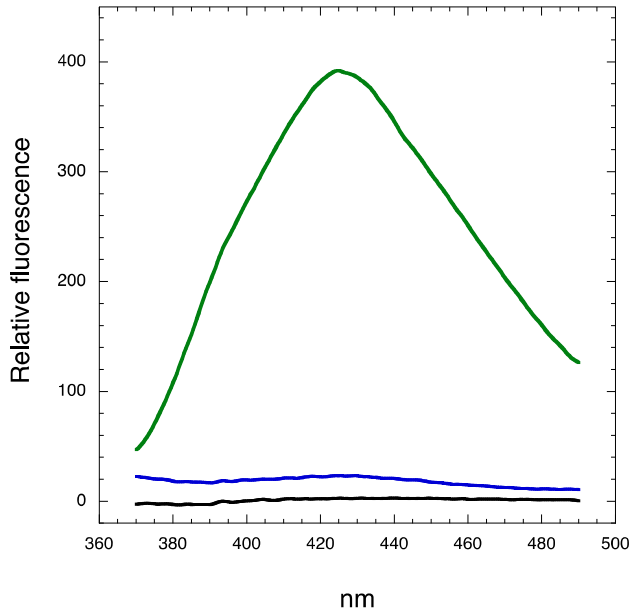


Figure 3. Fluorescence spectra of extensively washed *ec*PGDH and PGA. Black, PGA alone; Blue, *ec*PGDH alone; Green, *ec*PGDH with added PGA. Excitation wavelength is 340 nm.

To further test this observation, concentration dependent analyses were performed by varying either PGA or NAD^+. Figure 4 shows that there was substantial production of phosphoserine (as measured by phosphate production after addition of PSP) with no added NAD^+, as previously observed, and that the phosphoserine production increased only by approximately 20% with the addition of 1250 μM NAD^+ and

then remained level after that. Varying the concentration of PGA in the presence and absence of NAD^+ also showed only approximately a 20% difference in maximum level of phosphoserine produced. These observations indicate that the occupancy of the intrinsically bound NAD^+ was approximately 80% in the isolated and washed *ec*PGDH used in this example.

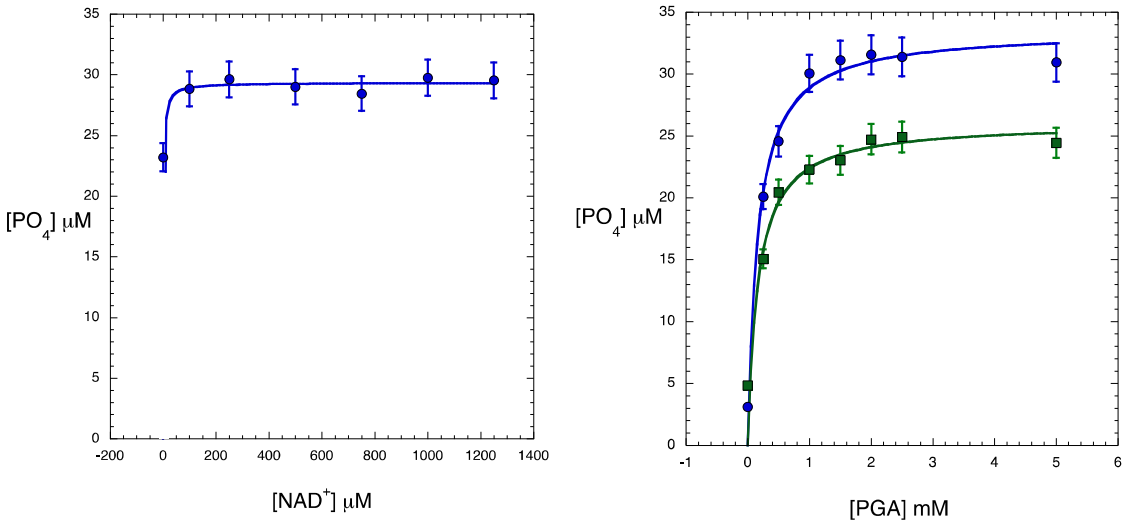


Figure 4. Concentration dependence of the reaction. Left: NAD^+ is varied from 0 to 1250 μM in the presence of 5 mM PGA and 200 mM Glu. Right: PGA is varied from 0 to 5 mM in the presence (③) and absence (⑤) of 1250 μM NAD^+ with 200 mM Glu in both. The reaction contained *ec*PGDH and PSAT. PSP was added after 10 min. Data is the average of duplicate determinations and error bars represent ± SEM. If error bars are not evident, they are within the area of the symbol.

In the experiments presented so far, PSP was added after the reactions were allowed to progress for 10 minutes. The experiments were designed in this way to prevent feedback inhibition of the *ec*PGDH by the serine produced. Figure 5 shows that if PSP is added at the start of the reaction, substantially less serine is produced (as measured by phosphate production from conversion of phosphoserine to serine plus phosphate). This is consistent with the *in situ* inhibition of the reaction by serine and verifies that the entire serine biosynthetic pathway is functional in these reactions.

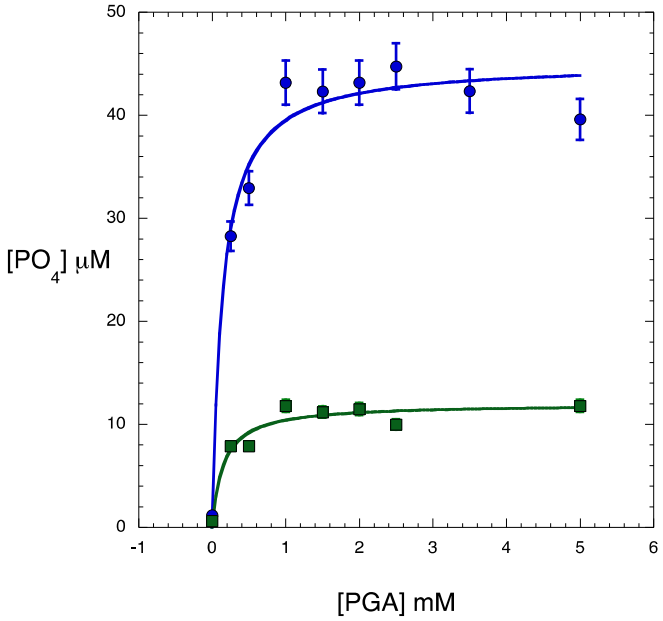
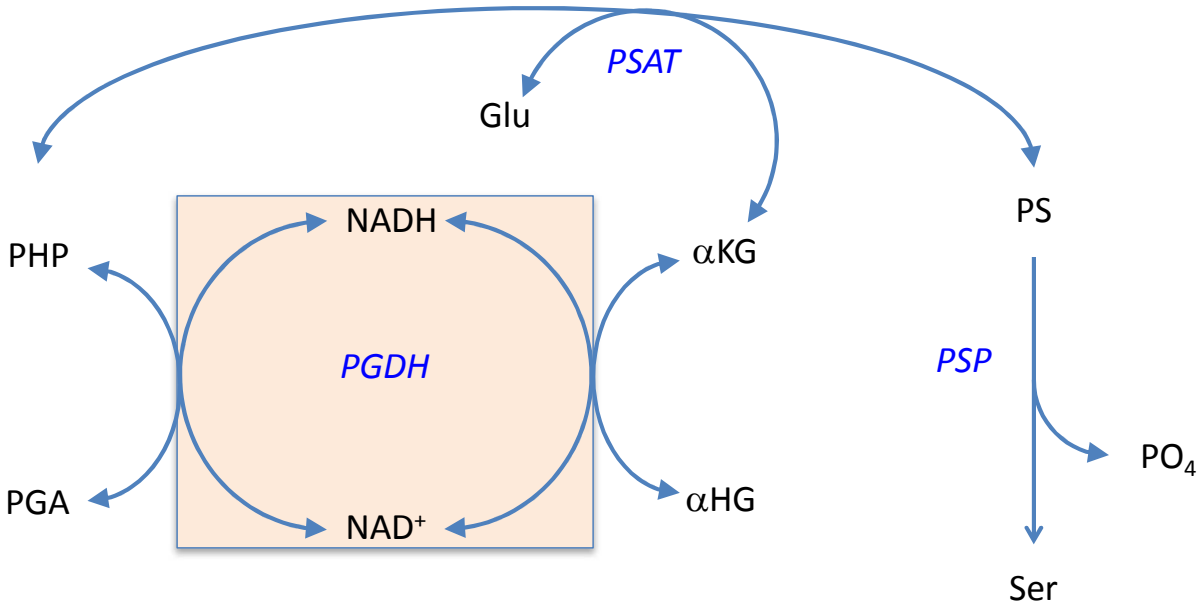


Figure 5. Concentration dependence of the reaction in the presence and absence of phosphoserine phosphatase at the beginning of the reaction. The reactions contained 1250 μM NAD^+ and 200 mM Glu in both as well as *ec*PGDH and PSAT. PGA is varied in the presence (⑤) and absence (③) of PSP at the start of the reaction. Data is the average of duplicate determinations and error bars represent ± SEM. If error bars are not evident, they are within the area of the symbol.

DISCUSSION

A simple attempt to monitor the activity of PGDH in the forward reaction direction by coupling its activity to PSAT resulted in several unexpected observations that further emphasize the differences in serine metabolism among species. Two major conclusions are evident. First, *ec*PGDH conserves coenzyme in the production of L-serine by utilizing an intrinsic cycle of NAD^+/NADH interconversion that can be maintained by production of αKG by the second enzyme in the pathway, PSAT (Scheme 2). This is not the case for PGDH from another bacterial source, *M. tuberculosis*, and a mammalian source, human liver, where net consumption of NAD^+ occurs. Second, both NAD^+ and NADH remain bound to *ec*PGDH during the cycle, effectively removing a requirement for the presence of an exogenous coenzyme pool to maintain the pathway. That NADH remained tightly bound to *ec*PGDH was already known, but this is the first demonstration that the same is true for NAD^+.



Scheme 2. The proposed NAD^+/NADH cycle in the production of L-serine by *ec*PGDH. PGA, D-3-phosphoglycerate; PHP, phosphohydroxypyruvate; PS, phosphoserine; Ser, L-serine; NAD^+/NADH, nicotinamde adenine dinucleotide; Glu, L -glutamic acid; αKG, alpha-ketoglutarate; αHG, alpha-hydroxy glutarate; PGDH, D-3-phospoglycerate dehydrogenase; PSAT, phosphoserine aminotransferase; PSP, phosphoserine phosphatase. The box represents *E. coli* PGDH with bound NADH and NAD^+ as isolated.

Thus, the ability of *ec*PGDH to utilize αKG as a substrate provides for a mechanism where biosynthesis of L-serine proceeds efficiently without a net consumption of NAD^+. In addition, for every molecule of PGA consumed a molecule of αKG is formed by PSAT, the second enzyme in the pathway. Under the conditions of the reaction used in this investigation, the level of αKG produced by PSAT is sufficient to maintain the cycle. As a consequence of this, although αKG is not strictly required to initiate serine synthesis as suggested by Gusyatiner and Ziyatdinov, its conversion to αHG is an indispensable reaction in maintaining the production of L-serine in *E. coli*. The intracellular concentration of αKG in *E. coli* has been measured at 440 μM (45) and the K_m for αKG in phosphate buffer is 88 μM (18). So, although the data suggest that an αKG pool is not required to initiate the cycle, there is ample αKG *in vivo* to drive the cycle. This cycle does not occur with *tb*PGDH and presumably other enzymes that cannot utilize αKG as a substrate.

A question that is relevant to this observation is whether or not the enzymes in the pathway form a complex that allows the efficient transfer of αKG from one to another without being released into solution. The fact that PSAT from *E. coli* and *M. tuberculosis* can be used interchangeably *in vitro*, without any appreciable difference in the results, would seem to suggest not. However, one cannot dismiss the possibility that there has been an evolutionary conservation of the structural elements that function in the formation of a putative enzyme complex between PGDH and PSAT. Studies are ongoing to address this.

*ec*PGDH is isolated with essentially irreversibly bound, but sub-stoichiometric amounts of NADH, and the remaining subunits that do not contain NADH, contain NAD^+ and catalyze the conversion of PGA to PHP. In this respect, the tightly bound coenzyme in *ec*PGDH acts somewhat like a prosthetic group in that it is essentially irreversibly bound to the enzyme as opposed to being transiently associated with the enzyme as is usually the case for NADH with dehydrogenases. However, since the reverse reaction utilizing either αKG or PHP in the presence of NADH is robust and continuously turns over, resulting in net consumption of NADH, it would appear that NADH is capable of displacing bound NAD^+ while it has been shown that the opposite is not the case (18).

*ec*PGDH can be viewed as a dimer of dimers, where residues from each subunit of the dimer contribute to the catalytic site. Previous studies (44) used engineered asymmetric hybrid tetramers to demonstrate that the catalytic dimers can act independently of each other. Therefore, the mechanism is likely one where two active sites can catalyze the conversion of PGA and NAD^+ to PHP and NADH, while the other two can catalyze the conversion of αKG and NADH to αHG and NAD^+ and vice-versa as the reactions proceed. This proposal is illustrated schematically in Figure 6.

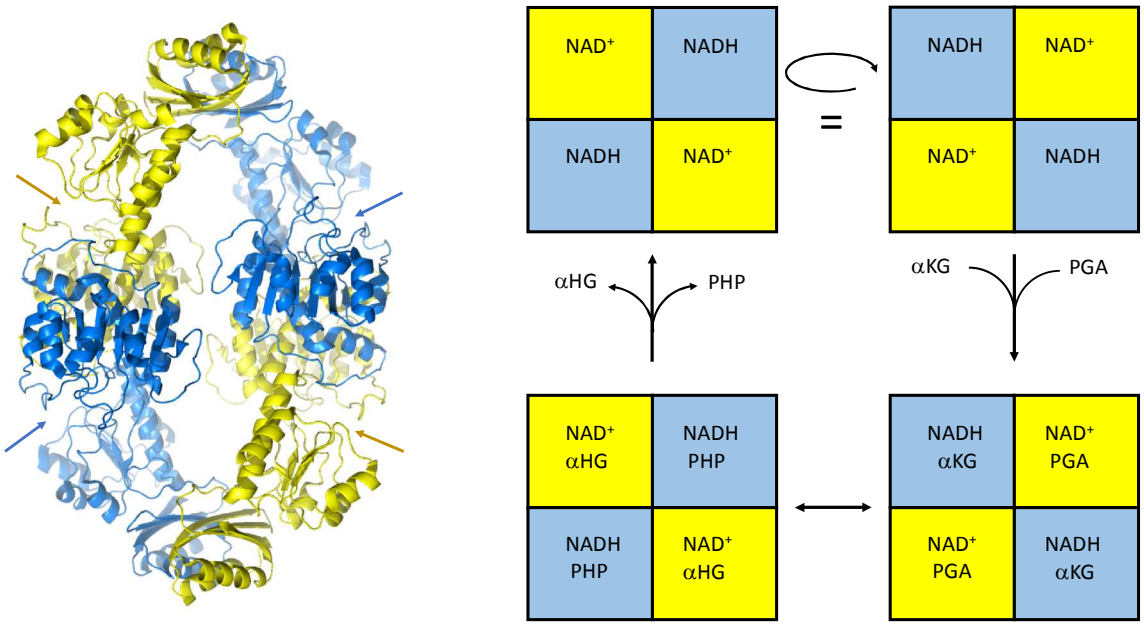


Figure 6. The *ec*PGDH tetramer is illustrated as a ribbon diagram (PDB: 1YBA) on the left with the arrows indicating the active site clefts. The subunits are colored alternately yellow and blue. On the right is a diagram depicting how the subunits may function in a flip-flop manner to simultaneously catalyze the conversion of the substrate pairs, NADH/αKG and NAD+/PGA. Note that since the tetramer is symmetrical, the top two representations are equivalent and can be superimposed with a 180º rotation.

Zhang et. al. recently made similar observations for PGDH from *Pseudomonas stutzeri* (19). That investigation showed that *ps*PGDH was capable of using αKG as a substrate, as had been previously reported for *ec*PGDH, and suggested that a similar cycle may be operative. However, they concluded that PGA was poorly oxidized in the absence of added αKG, and that a 2-hydroxyglutarate dehydrogenase (D2HGDH) was required in Pseudomonas species for efficient PGA oxidation. On the contrary, our data clearly show that the αKG generated by PSAT, the next enzyme in the pathway, was sufficient to produce phosphoserine at levels comparable to that produced by *tb*PGDH, an enzyme that does not utilize αKG,

under similar conditions when NAD^+ is supplied to the latter enzyme. Thus, there is no indication that a D2HGDH enzyme is required in *E. coli*. This is also consistent with the observation by Zhang et. al. that *E. coli* does not appear to contain a gene that codes for a similar D2HGDH.

The reactions catalyzed by PGDH and PSAT *in vivo* would eventually reach an equilibrium state, but since the reaction catalyzed by PSP is irreversible, this enzyme acts as a sink to draw the entire pathway in the direction of L-serine production. As expected, when all three enzymes of the L-serine biosynthetic pathway are present at the outset, a natural attenuation of the production of L-serine is accomplished by the feedback mechanism wherein L-serine inhibits the activity of PGDH. Under the conditions of the assay, a steady state level of L-serine is produced at the lowest concentration of PGA used. This is consistent with an apparent K_m of 0.15 ± 0.04 mM determined from the data in Figure 4. The concentration of L-serine produced, approximately 10 μM, is equal to the IC_{50} of L-serine for *ec*PGDH in non-phosphate buffers. Of course, L-serine will continuously be converted to other metabolites *in vitro*, so the situation will be much more dynamic.

The relatively large amount of assimilated carbon, estimated at 15% (15), that passes through L-serine when *E. coli* is grown on glucose would impose a large demand on the organism for NAD^+. This investigation demonstrates that *E. coli* has evolved to relieve this metabolic burden by a mechanism where it efficiently recycles NAD^+/NADH during the normal course of L-serine production by utilizing a metabolite that is naturally produced by the biosynthetic pathway itself, αKG. Furthermore, the cycle is essentially self-sustaining and is not dependent on any other enzymatic activity.

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CONFLICT OF INTEREST

The author declares that he has no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

GAG conceived and directed the study and wrote the paper

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Supporting Information. Supporting Information is available free of charge on the ACS Publications website.

A Figure showing the velocity versus substrate concentration plot for *SerA2*, a D-glycerate dehydrogenase (also called hydroxypyruvate reductase or glyoxylate dehydrogenase) is provided.

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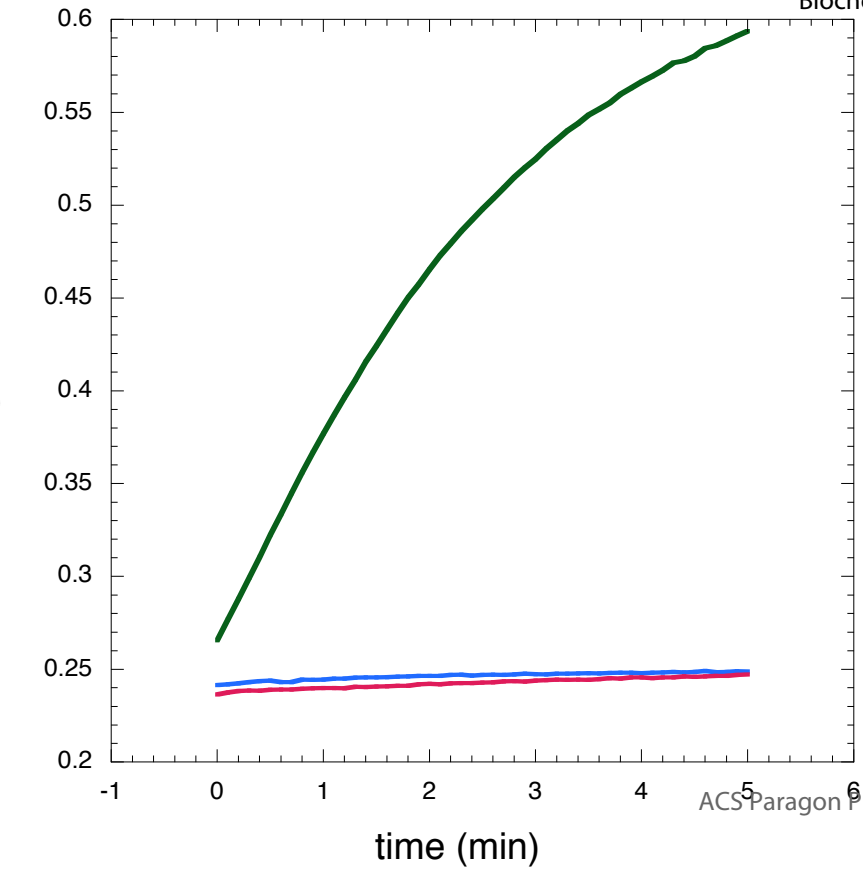

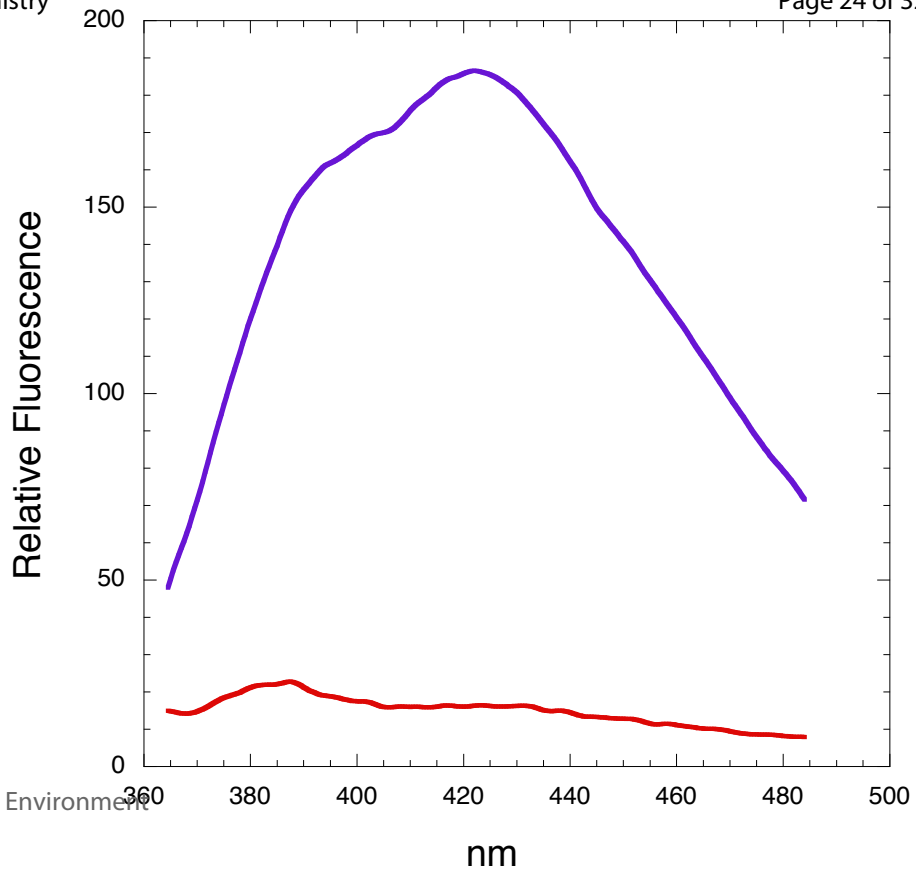

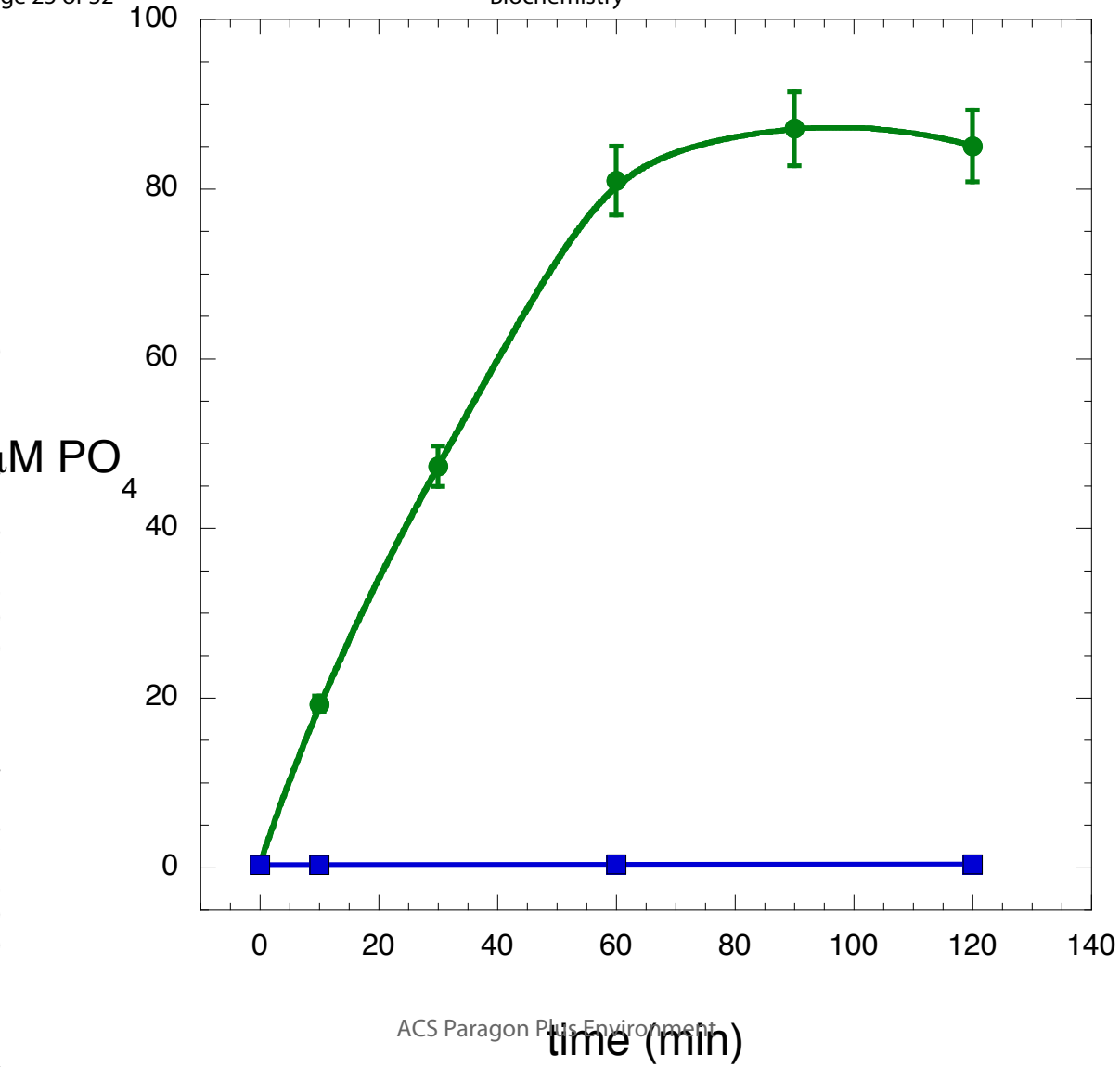

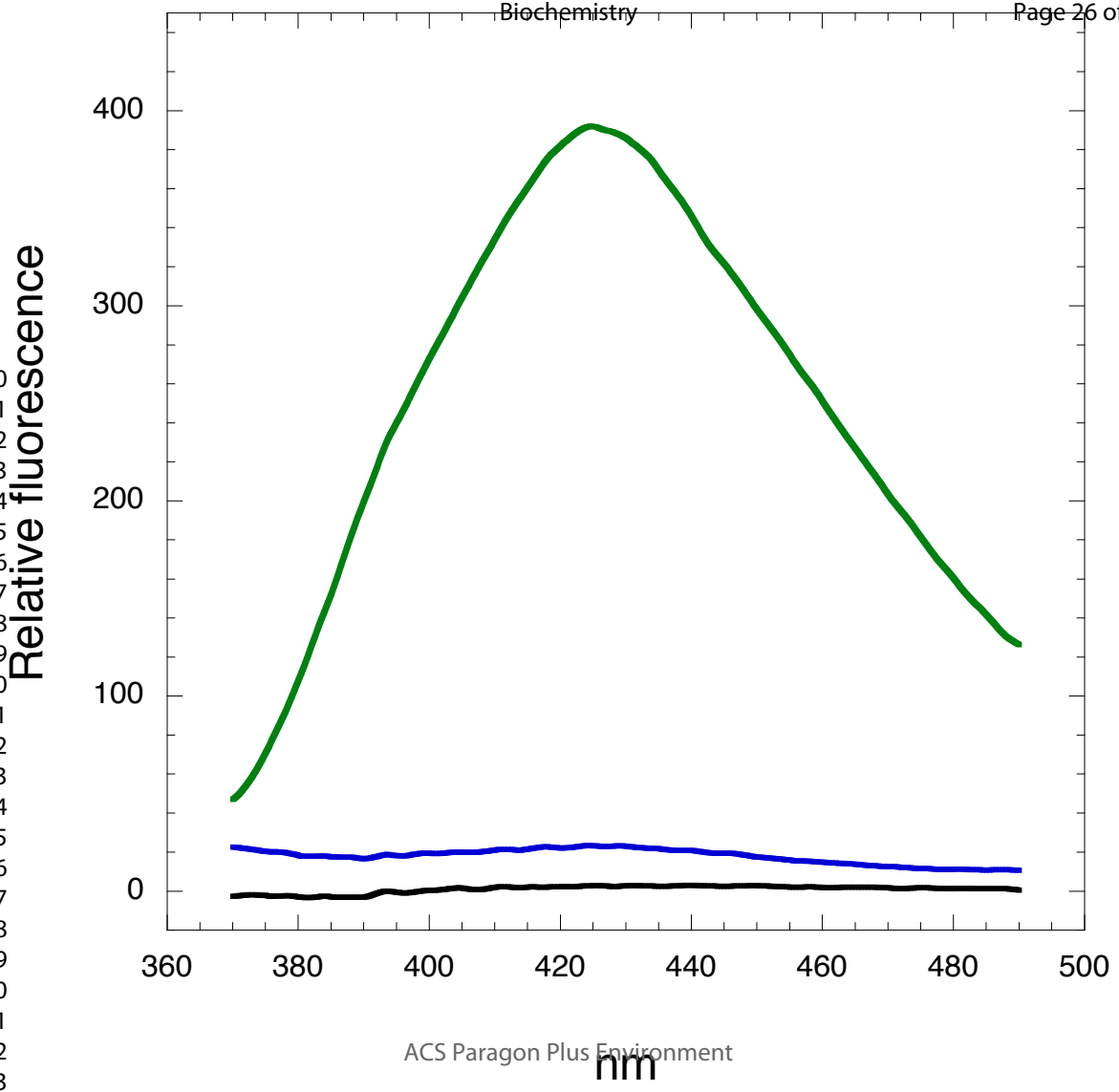

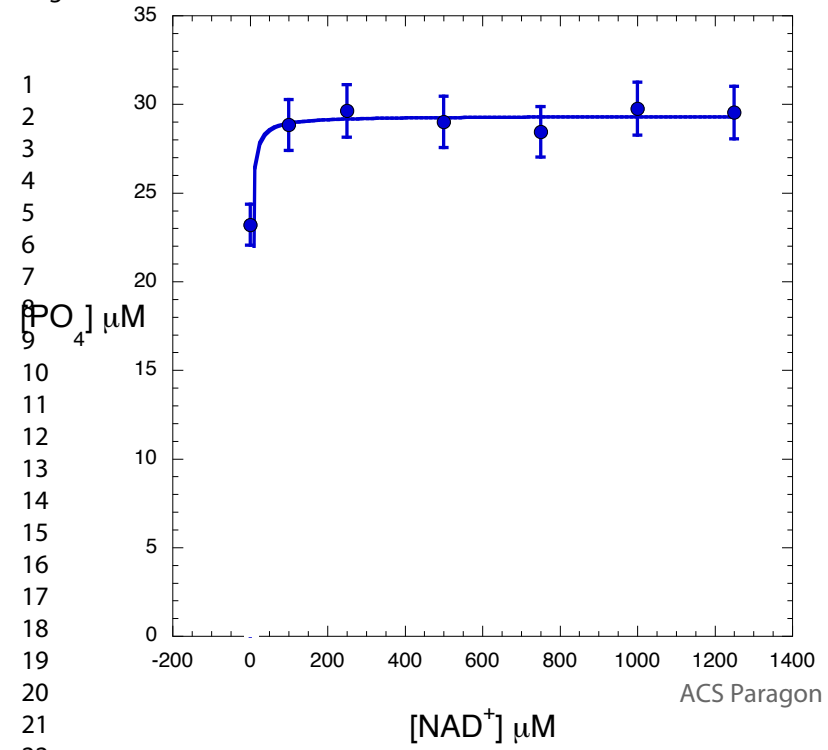

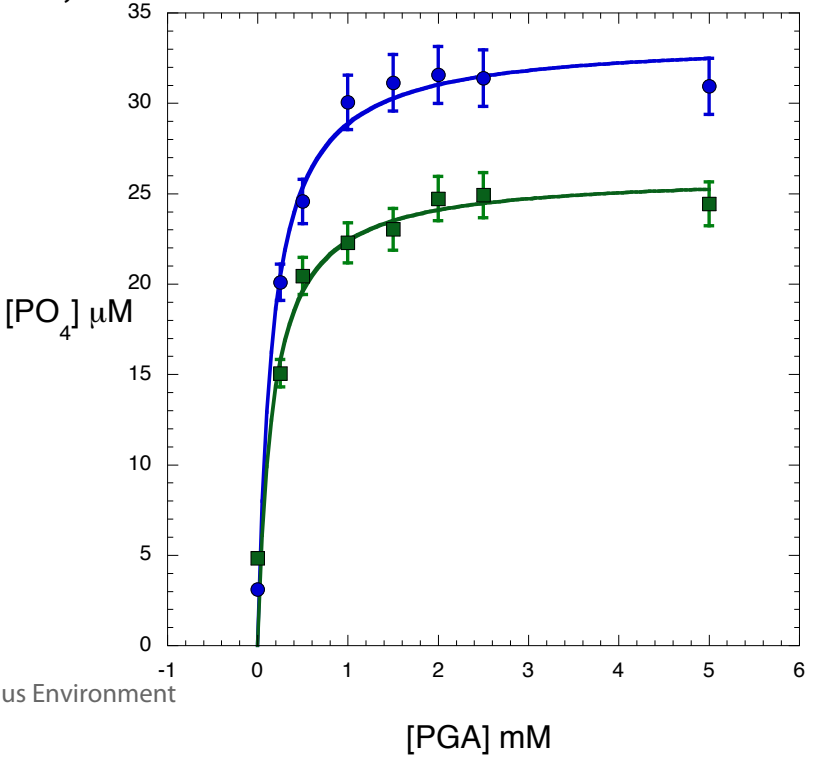

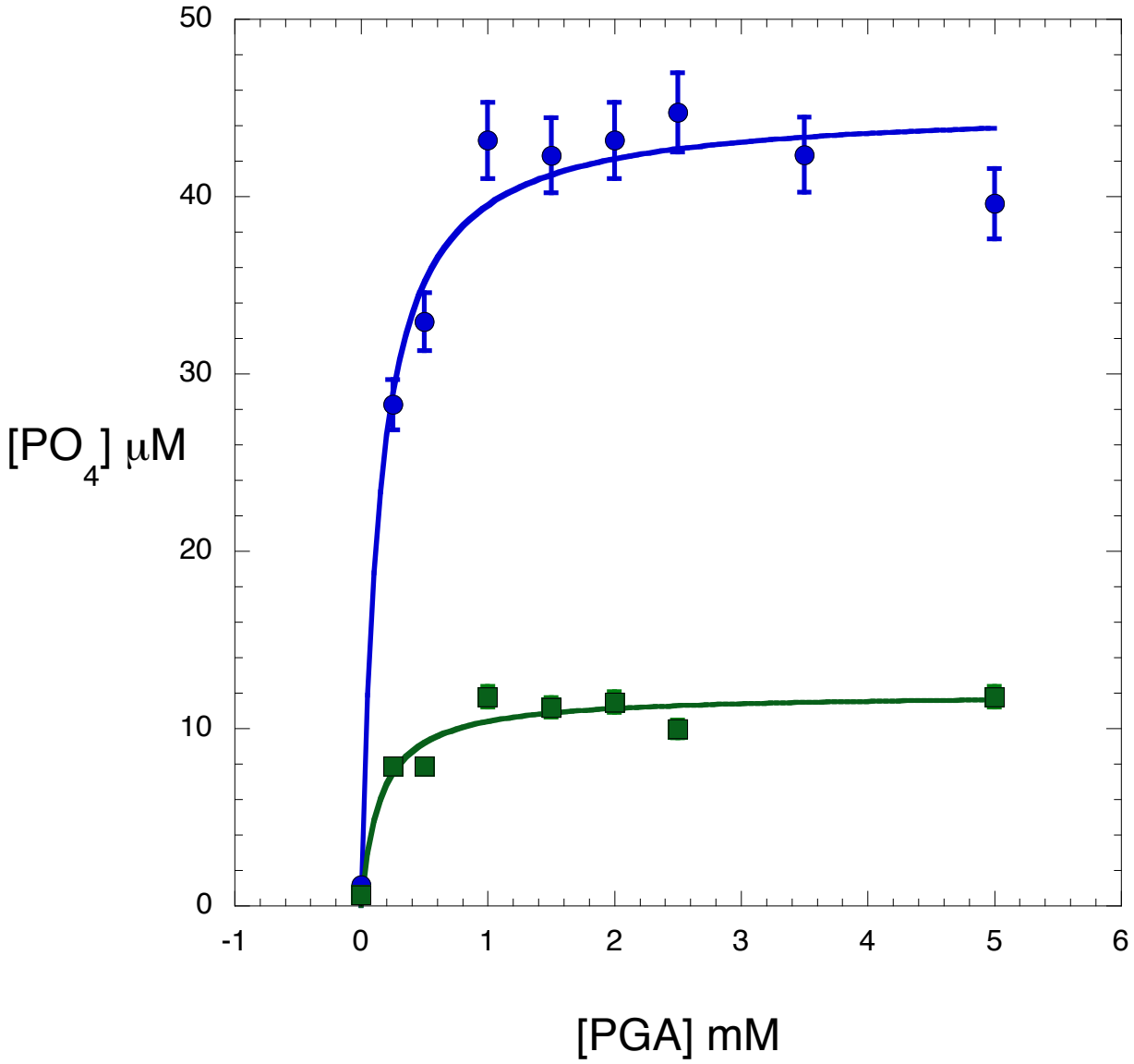
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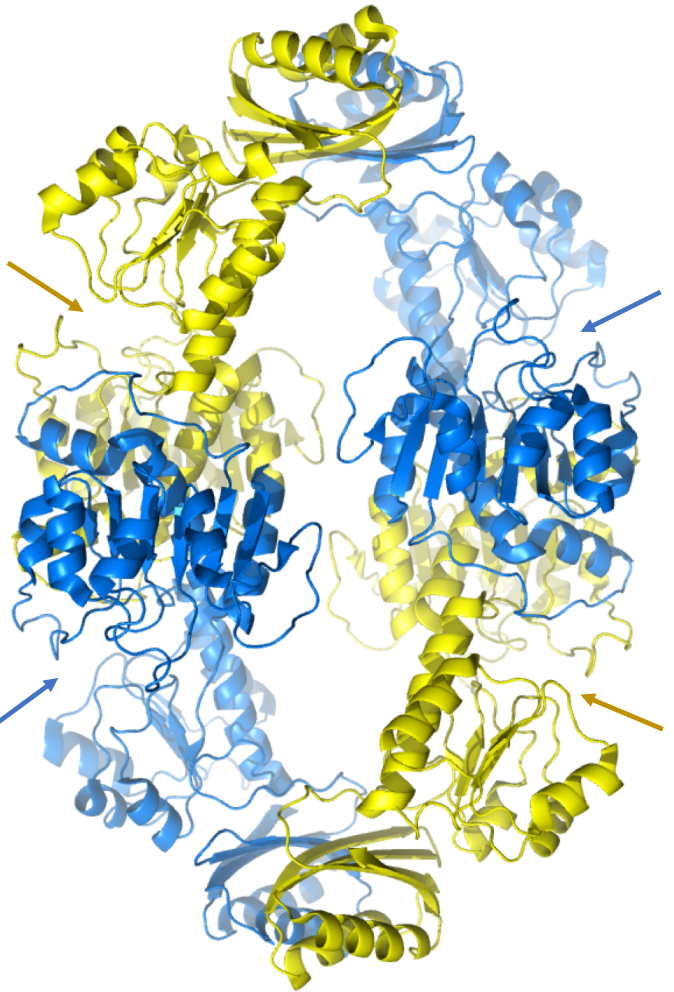

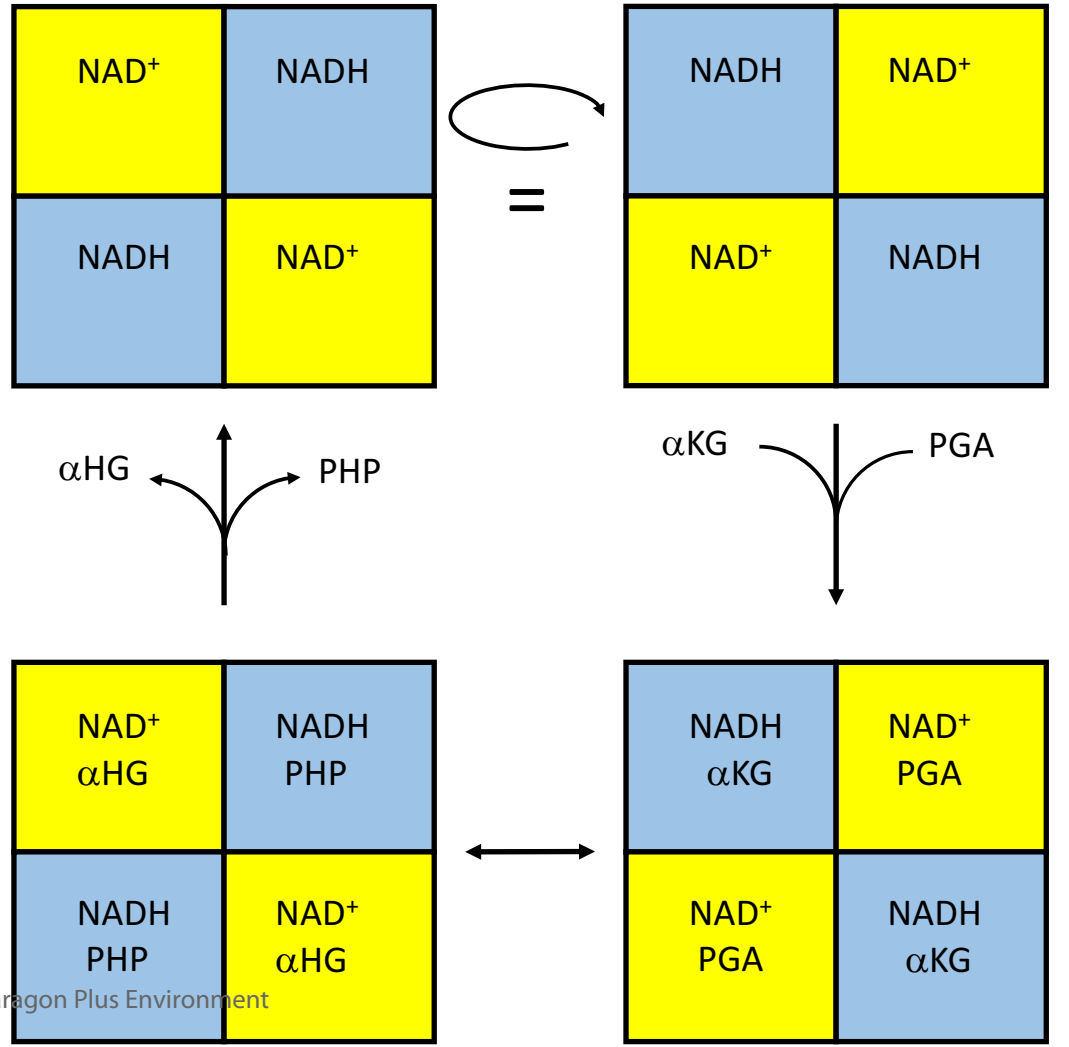

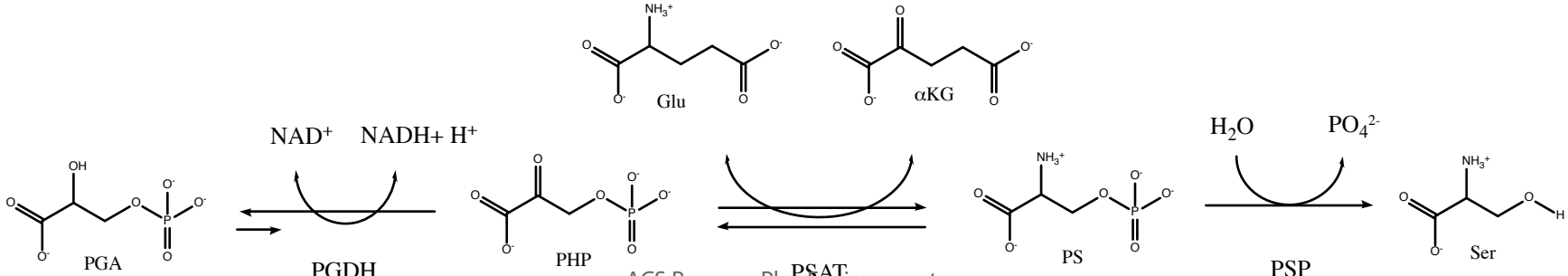

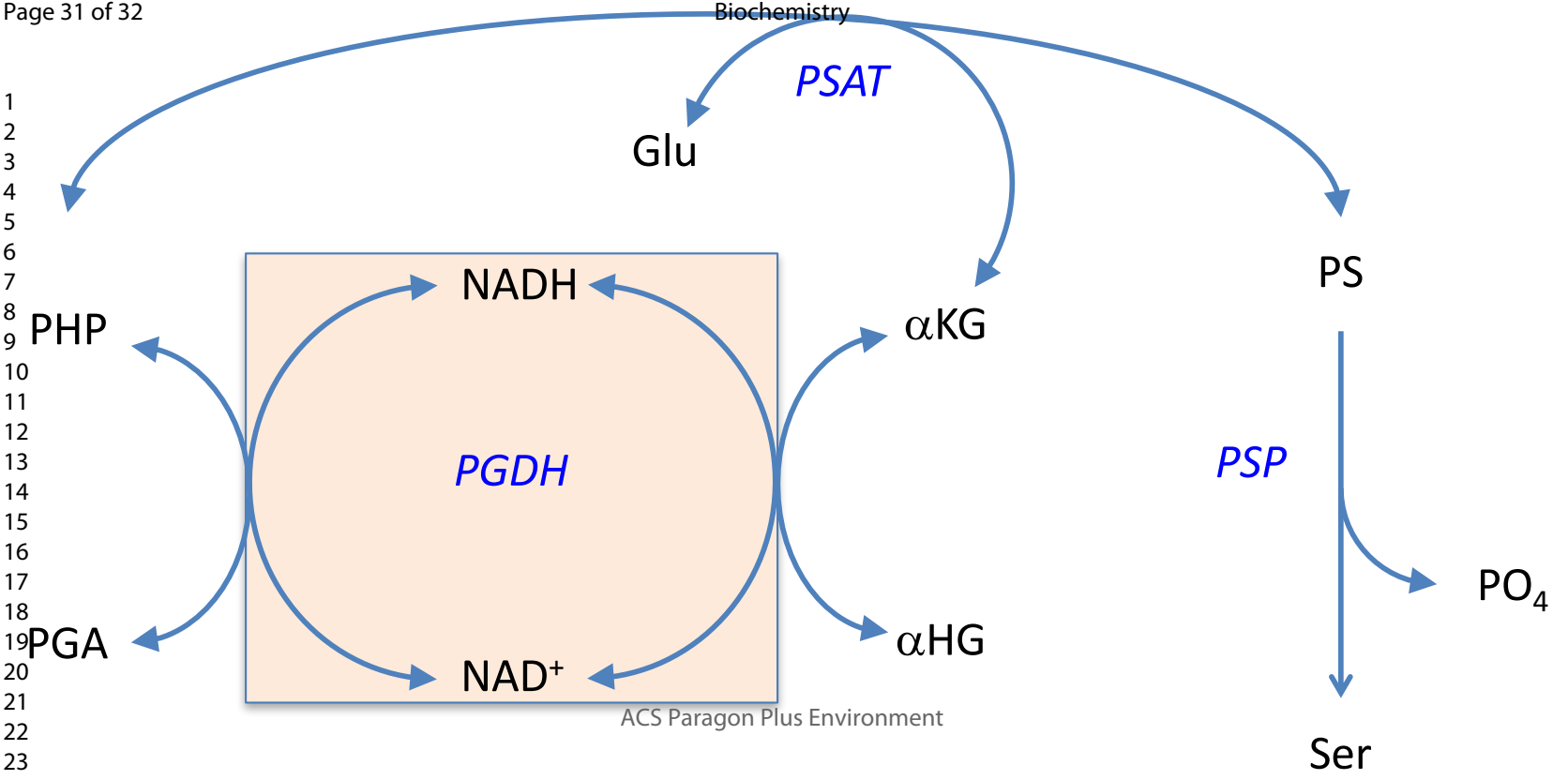

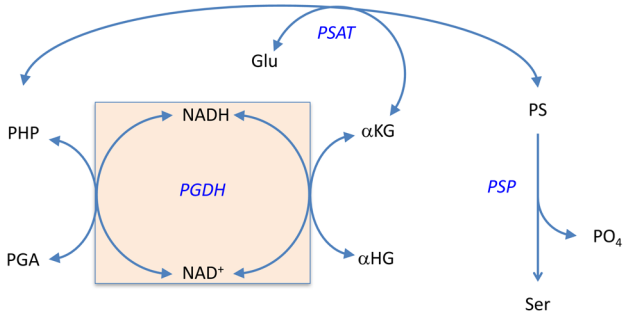
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