Membrane association of proline dehydrogenase in *Escherichia coli* is redox dependent

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**ABSTRACT** The PutA protein, product of the *Escherichia coli* gene putA, has two functions essential for proline utilization and for the regulation of *putP* and *putA* expression: as the peripheral membrane flavoprotein, proline dehydrogenase (EC 1.5.99.8), it transfers electrons from proline to the respiratory chain, and, as a repressor, it controls expression of genes *putP* and *putA* in response to proline supply. Association of proline dehydrogenase with the membrane was shown to require the simultaneous presence of the soluble enzyme, membranes, and proline. The kinetics of that association, monitored by following proline oxidation in a coupled enzyme assay system, were not altered when the transmembrane proton gradient generated during proline oxidation was dissipated by a proton ionophore. However, d-lactate or NADH could replace proline as a promoter of proline dehydrogenase–membrane association under anaerobic reaction conditions. These data imply that reduction of proline dehydrogenase and/or a membrane constituent promotes enzyme–membrane association. A biochemical mechanism is suggested whereby the concentration of proline dehydrogenase associated with the respiratory chain would be determined by proline supply.

Proline can serve as the sole source of carbon and nitrogen for bacterial growth. To utilize proline, *Escherichia coli* and *Salmonella typhimurium* must express the genes *putP* and *putA*, which are adjacent, divergently transcribed, and coinduced by proline (1-3). Gene *putP* encodes proline porter I, a Na+/proline symporter powered by the pmf (1, 4-6). In its role as the membrane-associated enzyme, proline dehydrogenase (EC 1.5.99.8), the PutA protein transfers electrons from proline to the respiratory chain (7-9). In addition to that enzymatic role, the PutA protein acts as a repressor controlling expression of *putP* and *putA* in response to proline supply (1, 2, 10, 11). Thus, within the cell, membrane-associated, free-cytoplasmic, and DNA-bound forms of the PutA protein are expected to coexist. Menzel and Roth (11) and Maloy and Roth (10) proposed that proline promotes association of proline dehydrogenase with specific membrane receptors. They suggested that the ensuing decreased occupancy of DNA operator sites would result in induction of genes *putP* and *putA*. Saturation of the available membrane receptors with newly synthesized PutA protein would then lead to accumulation of the soluble protein, saturation of the DNA control sequence, and restored repression of *put gene* expression.

Proline dehydrogenase is a flavoprotein associated with the cytoplasmic surface of the bacterial plasma membrane (2, 7, 9, 12, 13). It was purified from both *E. coli* (8, 14) and *Salmonella typhimurium* (9) by low ionic strength buffer/EDTA (14) extraction of the membrane fraction, and allotropic properties belonging to soluble or membrane-linked proline dehydrogenase were defined (7). Proline:O$_2$ oxidoreductase with properties identical to those of the endogenous, membrane-associated enzyme was reconstituted from purified proline dehydrogenase and inverted cytoplasmic membrane vesicles derived from *putA*–bacteria (14). Enzyme–membrane association was shown to occur only in the presence of the substrate and inducer l-proline. The activity reconstituted was a saturable function of enzyme concentration but it attained a level 20-fold higher than that observed in membranes isolated from *putA*–bacteria induced to utilize proline (14). Furthermore, bacteria containing multiple copies of gene *putA* yielded membranes bearing 4-fold higher activity than did wild-type strains after growth on proline (13, 15). Those observations were not consistent with the proposal that specific membrane sites are saturated with proline dehydrogenase during induction of *putA in vivo*.

Membrane association of proline dehydrogenase is clearly relevant to its function both as an enzyme and as a repressor. Previous data suggested that a colorimetric assay could be employed to monitor the kinetics of that association (14). That observation has been verified, and the association of proline dehydrogenase with inverted cytoplasmic membrane vesicles is shown here to be redox dependent.

**MATERIALS AND METHODS**

**Chromatography.** Chromatography was performed on a 25-cm Whatman DE52 column equilibrated with 150 mM NaCl, 100 mM Tris-HCl, pH 8.0, and 1 mM EDTA.

**Determination of *E. coli* strains employed were CSH4 (F* lac* trp lacZ rpsL thi), from Cold Spring Harbor Laboratory (1); JT31 (CSH4 putA1::Tn5), from this laboratory (1); and W2405 (CSH4 Δ*putPA*)101 srl300::Tn10 recA56 pLC43-41, also from this laboratory (13, 15). Plasmid pLC43-41, constructed by Clarke and Carbon (16), is plasmid ColEl

**Abbreviations.** oAB, o-aminobenzaldehyde; PSC, D'-pyrroline-5-carboxylic acid.
with an *E. coli* DNA fragment containing genes *putP* and *putA* inserted at the EcoRI restriction site.

**Enzyme and Membrane Preparation.** Bacteria were cultured (7), inverted cytoplasmic membrane vesicles were prepared (14), and proline dehydrogenase was purified (14) as described. The proline dehydrogenase preparations employed had specific activities in the range 0.9–1.6 units/mg.

**Protein Determinations.** The protein content of enzyme and membrane preparations was determined by the Peterson modification of the Lowry procedure (17), using bovine serum albumin as a standard.

**RESULTS**

The Coupled Enzyme Assay System. Proline:O₂ oxidoreductase was measured using the coupled enzyme assay system described in reactions 1 and 2:

proline + 1/2 O₂ = PSC + H₂O₂,  

PSC + oAB → chromophore,  

where PSC is Δ¹-pyrroline-5-carboxylic acid. In this system proline oxidation, catalyzed by membrane-associated proline dehydrogenase, requires electron transfer via the membrane-associated respiratory chain to molecular oxygen. (The activity of the soluble enzyme can be expressed only if an exogenous electron acceptor is provided.) Reaction 1 is made zero order with respect to its substrates by providing them in excess. It is effectively irreversible since the product of proline oxidation, PSC, is consumed in reaction 2. The spontaneous reaction of PSC with oAB, reaction 2, is first order with respect to PSC, which is rate limiting, and is made zero order with respect to oAB by providing oAB in excess. The rate of reaction 2 can be manipulated by varying the concentration of oAB. That reaction is effectively irreversible since equilibrium strongly favors the product chromophore.

If the rate of reaction 1, determined by enzyme concentration, is constant, the concentration of PSC will approach a steady state, and the rate of color development due to reaction 2 will accelerate, approaching a steady-state rate directly proportional to enzyme concentration. The steady-state concentration of PSC attained will depend on the relative rates of reactions 1 and 2; the time required to attain one-half that steady-state concentration and one-half the steady-state rate of color development, t₁/₂, will be equal to ln 2/k, where k is the pseudo-first-order rate constant for reaction 2.

I have used this assay system to monitor changes in the activity of proline dehydrogenase. If that enzyme activity increases during the assay period, both the approach to steady state and the steady-state rate of color development ultimately attained may be altered. For example, the rate of proline oxidation, reaction 1, may accelerate as soluble proline dehydrogenase associates with membrane fragments containing the respiratory chain. If that association reaction approaches completion at a rate much slower than that of reaction 2, the time required to attain one-half the steady-state rate of color development in the coupled enzyme assay system will be prolonged. Conversely, any process leading to increased proline dehydrogenase activity that is rapid compared to reaction 2 will be without influence on the approach to steady state in the coupled assay system; that approach will again be determined only by reaction 2. In this system, the relationship between the rate of enzyme activation and the rate of reaction 2 can be explored by varying the concentration of the excess reagent oAB in the reaction mixture. As long as the conditions outlined above are met, these effects on the approach to steady-state reaction conditions will not perturb the proportionality between the steady-state rate of color development approached and the enzyme activity attained during the assay.

**Proline Oxidation by Endogenous Proline Dehydrogenase.** Membranes prepared from *putA*⁺ bacteria grown on medium containing L-proline possess endogenous proline dehydrogenase. The half-time for approach to a steady-state rate of color development (t₁/₂) and the final rate approached (Vₖ) in the coupled enzyme assay were measured using membranes from strain CSH4 at eight concentrations in the range of 8–260 μg/ml. (The reaction mixture contained buffer, 5% (vol/vol) glycerol, 4 mM oAB, and 30 mM L-proline.) Both t₁/₂ and Vₖ, determined as illustrated in Fig. 1, were independent of the quantity of membrane protein (mean rate, 14 nmol/min per mg of membrane protein; mean t₁/₂, 66).

Further, no increase in the steady-state rate of color development was observed when the assay mixture was supplemented with membranes from strain JT31 (*putA*⁻), which lacks the enzyme (JT31 membranes added at 160 or 320 μg/ml to the assay system described above; data not shown).

To assess the adherence of the coupled enzyme assay system to the predictions stated above, the pseudo-first-order rate constant for reaction 2 was compared with the rate of approach to steady-state color development during enzymecatalyzed proline oxidation (reaction 1 plus 2). In each case, reaction kinetics were analyzed as a function of oAB concentration (Fig. 2). The pseudo-first-order rate constant for the reaction of PSC and oAB (reaction 2) was a direct function of oAB concentration as expected, yielding a value for the second-order rate constant of 8.4 s⁻¹·M⁻¹. That behavior was not matched by the enzymatic reaction.

The rate of approach to steady-state color development in the coupled enzyme assay approached a limit as oAB
Concentration increased. At 4.4 mM oAB that rate was only 49% of the corresponding rate for reaction 2. Thus the rate of approach to steady-state color development in this system was not determined solely by reaction 2. The data suggest that proline dehydrogenase undergoes a proline-dependent activation step whose rate is similar in magnitude to that of reaction 2 when oAB is at 4 mM. The solubility of oAB is limited, and proline dehydrogenase is inhibited by prolonged exposure to high concentrations of that reagent (data not shown). Thus other approaches will be required to further explore this activation phenomenon, which may prove analogous to the well-documented activation of succinate dehydrogenase (18). That these observations do not preclude use of the coupled enzyme assay system to analyze proline dehydrogenase–membrane association is shown below.

**Reconstitution of Proline:oAB Oxidoreductase.** The kinetics of proline oxidation were monitored in a series of reaction mixtures containing membranes from strain JT31 (putA−) and purified proline dehydrogenase (Fig. 3). The steady-state rate of color development approached (Vp) and the t1/2 for approach to that rate were determined graphically as illustrated in Fig. 1. The steady-state rate of color development approached was a nonlinear function of soluble enzyme concentration (membrane concentration constant, Fig. 3A)

![Graph](image-url)
enous enzyme would be the same as those observed during reconstitution, and the activity attained would increase as increasing quantities of the membrane preparation were added to the assay mixture. On the same basis, proline oxidation would be increased by the addition of membranes lacking proline dehydrogenase. Since neither of those effects was observed, the membrane preparation must be devoid of soluble enzyme, and the membrane-bound form of proline dehydrogenase must be strongly favored over the soluble form with which it is in equilibrium. Crude cell lysates from bacteria induced to utilize proline contain significant quantities of both soluble and membrane-bound proline dehydrogenase (L. G. Baker, S. B. Graham, J. T. Stephenson, J. M. Tham, and J. M. W., unpublished data). The soluble enzyme detected in those preparations may, therefore, be proline dehydrogenase that has not yet associated with the cytoplasmic membrane, not enzyme shed from the membranes during their isolation.

The Redox Dependence of Reconstitution. Membrane binding of proline dehydrogenase and reconstitution of proline:O₂ oxidoreductase activity were shown to require the simultaneous presence of membranes, enzyme, and proline (14). That observation implied that electron transfer or generation of a pmf was required to promote enzyme–membrane association. Carbonyl cyanide 3-chlorophenylhydrazone (5 μM) dissipated the proton gradient generated in inverted bacterial cytoplasmic membrane vesicles during proline oxidation (7), and at 10 μM it had no effect on the kinetics of proline oxidation catalyzed by endogenous proline dehydrogenase or during reconstitution of that activity (data not shown). Incubation of the reconstitution mixture with D-lactate (6 mM) under the standard, aerobic assay conditions for 30 min before initiation of the reconstitution reaction with proline was also without effect on reconstitution kinetics.

To verify that a concentrated membrane suspension incubated without aeration in the presence of an electron donor would become rapidly depleted of oxygen, the following experiment was performed. A 1-ml stoppered reaction mixture in a cuvette contained membrane protein (2.2 mg from strain WG2405; NADH dehydrogenase activity, 0.2 μmol/min per mg of protein), NADH (0.52 mM), carbonyl cyanide 3-chlorophenylhydrazone (10 μM), oAB (4 mM), glycerol (0.5%), and catalase (50 μg). Proline (30 mM) was added, and the rate of proline oxidation was observed by monitoring the absorbance change at 443 nm. Proline oxidation ceased after a 10-min incubation at 25°C. Addition of hydrogen peroxide at that time yielded an increase in absorbance that indicated stoichiometric O₂ evolution and proline oxidation.

That mechanism was used to attain membrane association of proline:O₂ oxidoreductase in the absence of proline. Membranes were mixed at high concentration in the reaction buffer with soluble proline dehydrogenase. Those destined to undergo depletion were stoppered and flushed with N₂, D-lactate or NADH was added to the N₂-flushed vials, and, at a series of subsequent times, aliquots of the membrane suspension were transferred to aerobic reaction mixtures containing buffer, oAB, and L-proline. The kinetics of proline oxidation were monitored (Fig. 4). The half-time for approach to a steady-state rate of color development in the aerobic reconstitution mixture did not vary. That for the systems incubated with NADH or D-lactate decreased steadily, implying that proline dehydrogenase membrane association was complete before initiation of proline oxidation with proline. The steady-state reaction rates attained in the mixtures to which NADH or D-lactate was added were higher than that attained in the aerobic reconstitution mixture. Elevated activities would be expected if enzyme–membrane association were completed in the precubination mixture when both enzyme and membranes were present at high concentration.

Fig. 4. Reduction promoted proline dehydrogenase–membrane association. Proline oxidation was determined by the oAB assay as described in Fig. 1 using the Unicam SP1700 Spectrophotometer. Membranes from strain JT31 (6.6 mg of membrane protein per ml) were mixed with purified proline dehydrogenase (0.77 unit/ml) in buffer plus 23% (vol/vol) glycerol under air (A) or N₂ (B and C). D-lactate (6 mM, B) or NADH (0.52 mM, C) was added to the N₂-flushed suspensions, and at a series of subsequent times (t, indicated by symbols ■, ○, and □), aliquots (6 μl) were transferred to a 1-m1 reaction mixture containing buffer plus L-proline (30 mM) and oAB (4 mM). The rate of proline oxidation (Vₜ) is plotted as a function of time (t) subsequent to the initiation of proline oxidation, the format illustrated in Fig. 1A.

DISCUSSION

These observations support the view that the rate of approach to steady-state proline oxidation in the oAB assay can be used to assess the rate at which proline dehydrogenase associates with membrane fragments (14). They are consistent with the following reaction sequence:

\[ E_0 + D_R \rightleftharpoons E_R + D_O, \]  \[ E_R + M \rightleftharpoons EM, \]  \[ M_O + D_R \rightleftharpoons M_R + D_O, \]  \[ E + M_R \rightleftharpoons EM, \]  \[ EM \rightleftharpoons EM^*, \]  

\[ \text{proline} + O_2 \rightleftharpoons \text{P5C} + \text{H}_2\text{O}, \]  \[ \text{P5C} + oAB \rightarrow \text{chromophore}, \]
where E = the soluble proline dehydrogenase, the PutA protein; M = the membrane association site for E; EM = the proline dehydrogenase–membrane complex; EM* = the activated complex; D = an electron donor; O = oxidized; R = reduced.

The unusual feature of this reaction scheme is the redox requirement for membrane assembly of proline dehydrogenase. These experiments do not distinguish between a requirement for reduction of that enzyme (reactions 3A and 4A), a requirement for reduction of a membrane constituent (reactions 3B and 4B), and a requirement for both redox events. Although preincubation of proline dehydrogenase with proline prior to initiation of reconstitution with membranes did not accelerate enzyme–membrane association (14), the data in Fig. 3B suggest that the latter step would be rate determining at moderate concentration of the soluble enzyme whether or not it were prereduced. Menzel and Roth (19) demonstrated that the flavin moiety of purified proline dehydrogenase from _S. typhimurium_ can be reduced by proline _in vitro_. Scarpulla and Soffer (8) showed that _D-lactate_ competitively inhibits proline oxidation by purified proline dehydrogenase from _E. coli_ without itself serving as a substrate, and the PutA protein from _S. typhimurium_ has P5C:NAD* oxidoreductase activity (9). Thus the flavin is the probable target for reduction but, although _D-lactate_ and NADH are known to interact with the enzyme, further studies will be required to show whether electron flow from either compound to the PutA protein, either directly or via the respiratory chain, could occur under the conditions of my experiment. Clearly reduction of the respiratory chain would be achieved.

Hypotheses regarding PutA protein-mediated regulation have evoked saturation of specific membrane and DNA receptor sites by the PutA protein (10, 11, 14). PutA protein–membrane binding has not been fully defined, but my data show that proline dehydrogenase activities as much as 20-fold higher than those observed in membranes prepared from bacteria induced to utilize proline are attained by reconstitution (ref. 14 and Fig. 3). If the redox requirement for enzyme–membrane association reflects a need for reduction of a membrane constituent, the involvement of a specific membrane receptor is implied. On the other hand, nonspecific, redox-dependent association of the protein with membrane lipid would also explain my observations.

The redox state of proline dehydrogenase _in vivo_ is expected to be determined by the cytoplasmic concentrations of proline and _P5C_. Those concentrations will, in turn, reflect the extracellular proline supply, the activities of proline-specific transport systems in the cytoplasmic membrane, and _P5C:NAD* oxidoreductase_ activity. The latter activity, in _S. typhimurium_ at least, also resides in the PutA protein (19). _P5C_ is, therefore, unlikely to accumulate as long as _NAD* is available to effect its oxidation. A sensitive response to proline supply would be provided if the oxidized form of the soluble PutA protein were to bind a specific DNA sequence controlling _put_ gene expression whereas its reduced form were to associate preferentially with the cytoplasmic membrane as proline dehydrogenase.

Regulation of substrate utilization by competition among substrates for oxidation via the respiratory chain has been suggested but seldom demonstrated (20, 21). Such competition could occur at several levels. Direct competition among substrates is unlikely since multiple, specific dehydrogenases have been identified. Biochemical regulation of those enzymes in response to the supplies of an array of substrates may occur [for example, pyruvate, _D-lactate_, and _L-lactate_ are inhibitors but not substrates for proline dehydrogenase (8)]. Kung and Henning (20) reported competition among _D-lactate_, _L-lactate_, and alcoholic L-α-glycerophosphate dehydrogenases for association with the cytoplasmic membrane _in vivo_, but Haldar _et al._ (21) were unable to demonstrate competition among _D-lactate_, _D-amino_ acid, and _glycerol-3-phosphate_ dehydrogenases for association with liposomes or membrane vesicles _in vitro_. As they noted, competition among dehydrogenases for association with a common membrane receptor and competition among membrane lipid-associated enzymes for access to respiratory chain electron acceptors are distinct possibilities. Each could be effected via a redox switching mechanism like that suggested above for the PutA protein. Such a mechanism, if generalized, would modulate the proportions of various respiratory chain dehydrogenases associated with the cytoplasmic membrane in response to the supplies of their respective substrates. If the respiratory chain consists of independent enzymes or partial respiratory chain complexes whose mutual reactivity depends on their concentrations within the membrane phase, modulation of respiratory chain function in response to substrate supply could be explained without evoking the participation of a membrane receptor common to several enzymes.

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