Membrane association of sucrose synthase: changes during the graviresponse and possible control by protein phosphorylation

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Abstract Sucrose synthase (SuSy) plays an important role in sucrose degradation and occurs both as a soluble and as a membrane-associated enzyme in higher plants. We show that membrane association can vary in vivo in response to gravistimulation, apparently involving SuSy dephosphorylation, and is a reversible process in vitro. Phosphorylation of SuSy has little effect on its activity but decreases its surface hydrophobicity as reported with the fluorescent probe bis-ANS. We postulate that phosphorylation of SuSy (and perhaps other membrane proteins) is involved in the release of the membrane-bound enzyme in part as a result of decreased surface hydrophobicity.

Key words: Protein phosphorylation; Sucrose synthase; Surface hydrophobicity; Membrane association; Gravity response; *Zea mays* L. pulvinus

1. Introduction

Sucrose synthase (SuSy, EC 2.4.1.13) catalyzes a reversible reaction in vivo [1]; however, the enzyme is thought to function primarily in the direction of sucrose cleavage in plant sink tissues supplied with ample sucrose substrate and with a high demand for carbon in biosynthetic and respiratory pathways. The critical role played by SuSy in potato tubers was demonstrated by the observation that antisense inhibition of SuSy results in a dramatic reduction in tuber starch content [2]. In general, it is thought that the regulation of SuSy activity involves control of the steady state level of enzyme protein [3,4] and the concentration of hexose sugars, which inhibit the cleavage reaction in vitro [5]. Recently, it was found that some of the SuSy protein is associated with the plasma membrane and it was postulated that it may exist in a complex with β-glucan synthases to channel UDP-glucose into glucan (e.g. cellulose) synthesis [6–8]. SuSy has been shown to be phosphorylated in maize leaves [9] and soybean nodules [10]. In maize, a single serine residue is phosphorylated, which was identified as Ser-15 of the maize SS2 protein [9]. Phosphorylation was reported to stimulate the cleavage reaction, but other effects of the covalent modification are likely. The overall objective of the present study was to determine whether the membrane association of SuSy is dynamic, and if so, whether protein phosphorylation might affect the distribution of the enzyme between the soluble and membrane-associated forms. We show that gravity-induced differential cell elongation leads to an increase of membrane-associated SuSy protein, and that phosphorylation of the membrane-associated enzyme causes release from the membrane while dephosphorylation of the soluble enzyme promotes membrane association. Increased exposure of hydrophobic surfaces occurs in response to dephosphorylation, as revealed by binding of the fluorescent probe bis-ANS, may be part of the underlying mechanism.

2. Materials and methods

2.1. Materials

Maize (*Zea mays* L. cv Pioneer 3183) plants were grown in a soil mixture in a greenhouse and fertilized three times weekly with a modified Hoagland's solution. For gravistimulation, 5–6 week old maize plants were placed horizontally. The upper and lower halves of bending pulvini were harvested 2 days later directly into liquid nitrogen and stored at −80°C. [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA, USA); microcystin-LR was purchased from Calbiochem (La Jolla, CA, USA); Complete (protease inhibitor cocktail) was obtained from Boehringer (Mannheim, Germany) and all other biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Extraction and differential centrifugation

Plant tissue was ground in liquid nitrogen and homogenized with 4 volumes extraction buffer (50 mM MOPS-NaOH, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 2 mM DTT, 1 mM EDTA, 0.1 μM microcystin-LR, 1 mM PMSF, 0.05% (v/v) Brij 35 and 2.5% (v/v) Complete). The homogenate was filtered through Miracloth (Calbiochem) and centrifuged at 10 000 × *g* for 20 min. The supernatant (clarified) was centrifuged again at 100 000 × *g* for 1 h and the final supernatant (soluble fraction) was analyzed for SuSy activity and protein. The microsomal membrane pellet was washed with extraction buffer, resolubilized in extraction buffer with or without 1% (v/v) CHAPS (Sigma) and centrifuged at 100 000 × *g* for 1 h at 4°C to produce the supernatant (membrane) and pellet (particulate).

2.3. Desalting and assay of SuSy activity

Aliquots were desalted by centrifugal filtration on Sephadex-G25 into desalting buffer (50 mM MOPS-NaOH, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM $CaCl_2$, 0.1 mM microcystin-LR) and SuSy activity assayed immediately as described [2,9].

2.4. Protein quantification and Western blot analysis

Total protein content of fractions was determined according to the Bradford microassay (BioRad, Richmond, CA, USA). For Western blot analysis, proteins were subjected to SDS-PAGE and electroblotted onto Immobilon-P (Millipore). The protein blots were immunostained with mouse anti-maize sucrose synthase monoclonal antibodies, kindly provided by Dr. Prem Chourey. Immunodetection was performed with alkaline phosphatase-conjugated affinity purified

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Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonic acid; bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid; AP_5A, P^1,P^5-di-(adenosine-5′)pentaphosphate; PKA, catalytic subunit of cAMP-activated protein kinase; PMSF, phenylmethylsulfonyl fluoride; PP, phosphatase; Suc, Sucrose; SuSy, sucrose synthase

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goat anti-mouse antibodies followed by chemiluminescence detection according to the manufacturer's protocol (Tropix, Bedford, MA, USA). Immunostained SuSy protein was quantified by densitometry (Personal Densitometer SI, Molecular Dynamics; ImageQuant, Microsoft). Arbitrary units reflect differences in the intensity of chemiluminescence on the same membrane with limiting concentrations of primary antibody.

2.5. *In vitro phosphorylation/dephosphorylation of clarified crude extract*

Bending pulvini were extracted as described above without microcystin-LR. Aliquots of the clarified crude extract were incubated for 30 min at 30°C in buffer (50 mM MOPS, pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, 1 mM $CaCl_2$) under phosphorylating ('PKA': plus 0.1 μM microcystin-LR, 20 mM NaF, 50 U protein kinase A, 0.1 mM AP_5A, 2 mM ATP), phosphorylation status conserving conditions ('Control': plus 0.1 μM microcystin-LR, 20 mM NaF) or dephosphorylating conditions by endogenous protein phosphatases ('endo PP': no additions) or by alkaline phosphatase ('alk PP': 20 U alkaline phosphatase). After treatment, samples were separated into the soluble, membrane, and particulate fractions.

2.6. *In vitro and in vivo phosphorylation of the membrane-associated SuSy*

The microsomal membrane fraction was prepared from gravistimulated maize pulvini and resuspended in extraction buffer without detergent as described above. Aliquots were incubated under phosphorylating (0.1 mM AP_5A, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1.5 mM [γ-^{32}P]ATP (900 cpm/pmol)) conditions in the presence or absence of exogenous protein kinase (20 U PKA) or 10 mM sucrose. After 20 min at 30°C reactions were stopped by addition of 4 mM EGTA and samples fractionated into soluble (supernatant) and microsomal/particulate pellet by centrifugation at $100\,000 \times g$ for 1 h at 4°C. The pellet was washed twice, resuspended in detergent solution (50 mM MOPS, pH 7.5, 0.25% (v/v) CHAPS, 5% (v/v) Triton X-100), and the solubilized microsomal membrane separated from the particulate fraction by centrifugation as above. SuSy was immunoprecipitated from the soluble and membrane fractions [9], followed by SDS-PAGE and autoradiography. In vivo labeling of SuSy was carried out in 3 week old seedlings as described [9].

2.7. *SuSy purification and fluorometric determination of protein hydrophobicity*

SuSy was precipitated from the soluble fraction of extract of gravistimulated maize pulvini with 30% ammonium sulfate, affinity purified with an UDP-glucuronic acid agarose column [3], followed by Resource Q ion exchange chromatography using an FPLC system (Pharmacia). Peak fractions were pooled and desalted. Association of ANS or bis-ANS (Molecular Probes, Eugene, OR, USA) with SuSy (or a buffer control) was followed in a Fluorometer (Hitachi F2000) (excitation: $\lambda = 350$ nm; emission: scan λ 400 nm to 650 nm; time-courses: emission $\lambda = 500$ nm).

3. Results and discussion

3.1. *Effect of SuSy phosphorylation on the sucrose cleavage reaction*

We previously suggested [9] that phosphorylation of SuSy caused a significant activation of the Suc cleavage reaction, measured as Suc-dependent formation of UDP-glucose. However, at least part of the apparent activation of SuSy by pretreatment with ATP is artefactual. As shown in the first line of Table 1, SuSy can be inhibited completely by the phenylglycoside arbutin, as expected [11], but after ATP pretreatment arbutin no longer gives complete inhibition. Arbutin-sensitive UDP-glucose formation (third column in Table 1), the best measure of SuSy activity, was increased by ATP pretreatment but the real activation (0.7 nmol) was only about one-third of the apparent activation (2 nmol). The artefactual component (1.3 nmol) may involve UDP-glucose formation by UDP-glucose pyrophosphorylase in the crude extract. Thus, phosphorylation of SuSy does activate the cleavage reaction, but the effect is smaller than previously reported and may be insignificant physiologically. As discussed below, a major function of phosphorylation may be to control the intracellular localization of SuSy.

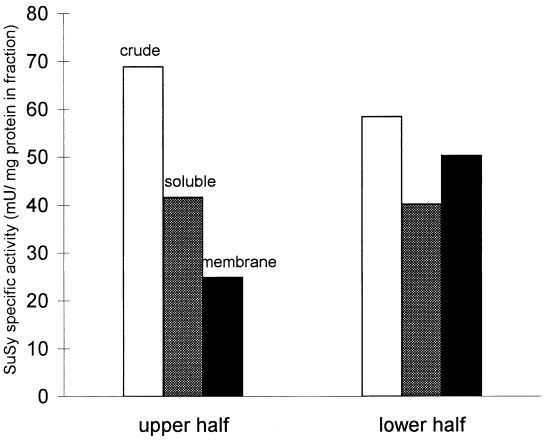

Fig. 1. Gravistimulation induces translocation of SuSy to the microsomal membrane fraction. SuSy specific activity (assayed with 100 mM Suc and 1 mM UDP, based on total protein/fraction) showed little change in the clarified crude or soluble fraction of gravistimulated maize pulvini (Upper, less elongating, and Lower, more rapidly elongating half) whereas SuSy activity associated with the microsomal membrane fraction in the upper half was only $60 \pm 11\%$ of that in the lower pulvinus portion ($n = 3$). In all tissue samples (upper and lower portion) membrane protein in the microsomal fraction accounted for only $2.2 \pm 0.2\%$ of the total protein in the crude extract.

3.2. *Gravity-induced cell elongation leads to increased membrane association of SuSy*

All grass stems contain a specialized region described as the pulvinus that is solely involved in gravitropism [12]. Maize is an example of a panicoid grass that has an internodal pulvinus at the base of each internode. The pulvinus is quiescent until gravistimulated and does not respond to other environmental signals such as light. For example, when a maize plant

Table 1
Increased synthesis of UDP-glucose after ATP pretreatment of desalted crude extract is partially independent of SuSy activity

	UDP-glucose formed (nmol/min)		
	−Arbutin	+Arbutin	Arbutin-sensitive
Control	2.6	0	2.6
ATP-pretreated	4.6	1.3	3.3
Apparent ATP activation	2.0	1.3	0.7

Assays were conducted with 10 mM sucrose, 0.37 mM UDP and 10 mM arbutin, as indicated. Arbutin-sensitive formation of UDP-glucose is taken as the best estimate of SuSy activity (representative experiment, $n = 3$). Arbutin-sensitive activation accounts for only $27 \pm 4\%$ of total apparent ATP activation.

becomes lodged (i.e. gravistimulated), the shoot bends upwards as a result of asymmetric growth within the pulvinus: cells on the lower side elongate more rapidly than those on the upper side. We have been studying changes in carbohydrate metabolism that accompany the gravitropically induced differential cell elongation in the maize stem pulvinus when plants are reoriented from vertical to horizontal. Interestingly, the total activity of SuSy in a pulvinus extract is unaffected by gravistimulation, as the specific activity of SuSy is nearly identical in the upper and lower portions of the graviresponding (horizontal) pulvinus (Fig. 1). However, in the faster elongating lower portion of the pulvinus there was always about twice as much SuSy protein and activity associated with the microsomal membrane fraction compared to the upper half. Since total membrane protein accounts for only $2.2 \pm 0.2\%$ of the total protein in each pulvinus extract, the increase in membrane-associated SuSy protein and activity is not reflected by a decrease of SuSy protein and activity in the crude fractions. Membrane-associated SuSy activity represents only 0.7% and 1.3% of the total SuSy activity in the crude fractions of upper and lower halves of gravistimulated pulvini, respectively. These results indicate that the association of SuSy with the membrane is dynamic in vivo.

3.3. *Differential phosphorylation of soluble versus membrane-associated SuSy in vivo*

As one approach to determine whether protein phosphorylation might be part of the mechanism controlling the localization of SuSy, we compared the soluble and membrane-associated proteins in terms of relative phosphorylation in vivo. For these experiments, we fed excised maize shoots [^{32}P]Pi via the transpiration stream and extracted SuSy from the leaf elongation zone [9] and compared the soluble versus membrane-bound enzymes. Of the total immunoprecipitated SuSy, the membrane-bound enzyme accounted for 9.9% of the SuSy enzyme protein but only 4.8% of the ^{32}P incorporated into SuSy. Thus, the soluble SuSy was more heavily phosphorylated than the membrane-associated enzyme. Consequently, experiments were conducted to determine whether phosphorylation could directly affect SuSy localization in vitro.

3.4. *Dephosphorylation of 'soluble' SuSy increases its association with membranes*

A clarified crude pulvinus extract (containing the microsomal membrane fraction and soluble proteins) was preincubated at 25°C in the absence (endogenous PP) or presence of protein phosphatase inhibitors (NaF and microcystin-LR; 'Control'), or with addition of alkaline phosphatase to facilitate dephosphorylation of proteins including SuSy. After preincubation, the distribution of SuSy protein between the soluble and membrane fraction was assessed by immunoblotting and activity assay. As shown in Fig. 2, pretreatment conditions that promoted protein dephosphorylation (endogenous PP and alk PP) reduced SuSy protein in the soluble fraction while increasing the protein firmly associated with the membrane fraction. When dephosphorylation was prevented with phosphatase inhibitors (microcystin-LR and NaF) the amount of membrane-associated SuSy protein and activity was lower than under dephosphorylating conditions. When phosphorylation was promoted by addition of ATP+PKA in the presence of phosphatase inhibitors, the membrane-associated SuSy protein and activity was reduced while SuSy protein in the soluble fraction was increased. The reciprocal changes in SuSy enzyme protein and activity distribution are consistent with the notion that protein dephosphorylation may mediate the translocation of SuSy to the membrane and that protein phosphorylation of SuSy is at least part of the mechanism that releases SuSy from the membrane.

3.5. *Phosphorylation causes release of membrane-bound SuSy*

In order to test whether phosphorylation of SuSy itself could be involved in its intracellular localisation, the microsomal membrane fraction was treated under phosphorylating

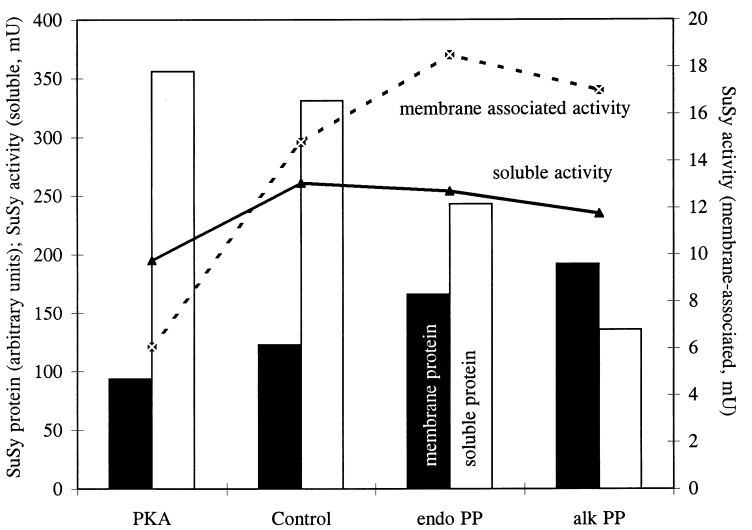

Fig. 2. Reciprocal changes in the distribution of SuSy between the membrane and soluble fractions as influenced by phosphorylation status. The phosphorylation status of proteins in a clarified crude extract was conserved by addition of the phosphatase inhibitors MC-LR and NaF in the absence of ATP (Control). In vitro dephosphorylation (endogenous PP and alkaline PP, no phosphatase inhibitors) increased the amount of SuSy protein and activity in the membrane fraction (black columns, dashed line) while SuSy protein in the soluble fraction (white columns) and activity (solid line) declined. Conversely, in vitro phosphorylation with PKA and ATP in the presence of phosphatase inhibitors (PKA) decreased the amount of membrane-associated SuSy protein (representative experiment, $n = 3$).

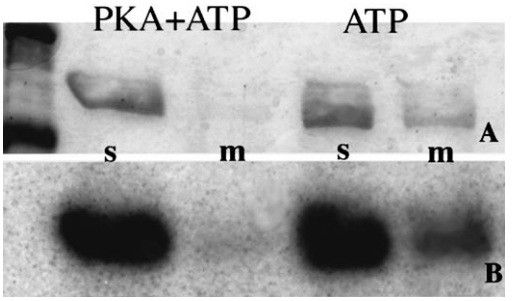

Fig. 3. In vitro phosphorylation of the microsomal membrane fraction leads to the release of SuSy protein into the soluble phase. Coomassie blue stained SDS-PAGE (A) and autoradiography (B) of the soluble (s) and membrane (m) fractions after immunoprecipitation show that in the presence of exogenous protein kinase (PKA), membrane-associated SuSy was almost entirely phosphorylated and released from the membrane into the soluble fraction. Endogenous protein kinase activity was apparently limiting since addition of ATP alone led only to a partial release of phosphorylated SuSy.

conditions in the presence of [γ-^{32}P]ATP and Ca^{2+}. Phosphorylation was terminated with EGTA and the soluble and membrane fractions separated. An autoradiograph of the immunoprecipitated SuSy showed that SuSy released from the membrane was phosphorylated (Fig. 3). Phosphorylation and release of phosphorylated SuSy protein from the membrane was greatest in the presence of PKA catalytic subunit. In the absence of PKA, less SuSy was released into the soluble fraction and at least part of the phosphorylated SuSy remained associated with the membrane. This suggests that either the activity of endogenous (membrane-associated) SuSy kinase is limiting and/or other membrane protein(s) have to be phosphorylated as well in order to release SuSy protein into the soluble fraction.

3.6. *Phosphorylation of SuSy affects binding of the hydrophobic probe bis-ANS*

One mechanism by which phosphorylation could affect its membrane association would involve conformational changes that affect exposure of hydrophobic residues. In fact, this notion is supported by our preliminary experiments with the hydrophobic fluorescent probe bis-ANS. In aqueous solution, the fluorescence of bis-ANS is very low, but in an apolar solvent or when associated with hydrophobic sites of a protein, fluorescence intensity is enhanced markedly [13,14]. When affinity-purified SuSy was incubated with bis-ANS, there was a time-dependent increase in fluorescence reflecting binding of the probe to the protein (data not shown). After about 30 min, the fluorescence emission was constant but an additional increase in fluorescence occurred after addition of alkaline phosphatase (Fig. 4A). Appropriate controls indicated that dephosphorylation of SuSy by the alkaline phosphatase was responsible for the fluorescence increase. Similar results were observed when the hydrophobic probe ANS was used instead of bis-ANS (data not shown). These results would suggest that dephosphorylation promotes a conformation change in which hydrophobic residues are more exposed to the solvent.

To further test this postulate, we attempted to increase the phosphorylation status of SuSy to a higher level by incubation with PKA+ATP. As shown in Fig. 4B, there was a time-dependent decrease in bis-ANS fluorescence when SuSy was mixed with ATP, PKA and Suc. The requirement for Suc for the fluorescence decrease is clearly shown in Fig. 4C. In other experiments, it was verified that all three components were required and that there was no decrease in fluorescence in the absence of SuSy, the presumed target of the fluorescent probe. The results support the postulate that phosphorylation caused a conformation change that results in decreased surface hydrophobicity, presumably by burial of hydrophobic side chains. The requirement for Suc suggests that substrate binding may be necessary for the presumed conformation change of phospho-SuSy to occur.

3.7. *Dephospho-SuSy is less soluble than phospho-SuSy*

During the course of our studies, we observed that the apparent solubility of SuSy was variable. After storage of fractions containing dephospho-SuSy at 4°C, samples developed a white precipitate (which could not be readily resolubilized) concurrent with the loss of SuSy protein from the soluble fraction. In contrast, fractions containing phospho-SuSy did not develop a protein precipitate and SuSy activity and protein content were stable at 4°C for several weeks. We postulate that dephospho-SuSy assumes a conformation where hydrophobic residues are exposed and when the concentration of protein is sufficiently high (as in peak column fractions), the hydrophobic areas form intermolecular contacts resulting in protein aggregation and precipitation. Although clearly not definitive, this observation is consistent

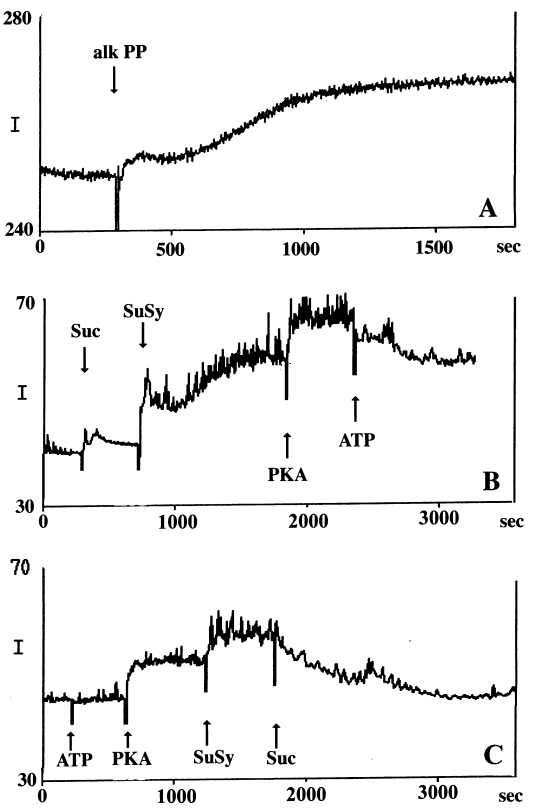

Fig. 4. Dephosphorylation of affinity-purified SuSy increases its hydrophobicity (A) while phosphorylation in the presence of 10 mM Suc leads to a decrease in the exposure of hydrophobic residues (B,C) of SuSy as monitored by bis-ANS fluorescence. The trace in (C) demonstrates the requirement for Suc for the decrease in bis-ANS fluorescence. Samples of purified SuSy were preincubated with 0.2 mM ANS or 0.1 mM bis-ANS for 1 h at 25°C in the dark (representative chart recordings, $n=2$ for A, $n=3$ for B, $n=2$ for C).

with the notion that phosphorylation affects SuSy structure in such a way that the exposure of hydrophobic groups is modulated.

4. Conclusion

There are two major conclusions that can be drawn from these studies. First, the membrane association of SuSy is shown to be dynamic in vivo, correlating with the extent of gravity-induced cell elongation. The increased membrane association may be necessary to support the increased cell wall deposition required for rapid cell elongation in the pulvinus (especially the lower surface). This would be entirely consistent with the notion that membrane-bound SuSy channels UDP-glucose formed during Suc cleavage to cellulose synthase [6,8]. The second major conclusion is that phosphorylation of SuSy may be at least part of the mechanism controlling SuSy localization, and is supported by three lines of evidence: (i) dephosphorylation of SuSy causes increased association with the membrane fraction; (ii) phosphorylation of membrane proteins caused the release of SuSy from the membrane; and (iii) in vivo phosphorylation studies indicate that the membrane-associated enzyme contains less ^{32}P than the soluble enzyme (SuSy protein basis). At least part of the underlying mechanism of reversible protein translocation may be phosphorylation-dependent conformation changes that affect surface hydrophobicity. That surface properties are altered is supported by studies with the hydrophobic probe bis-ANS and observations relating to differences in protein solubility.

Much remains to be done, but a new working model is forwarded that can now be tested. Clearly, one important question concerns identification of those residues whose exposure might be controlled by phosphorylation. It turns out that SuSy isoforms contain one (SS1) or two (SS2) $\sim$20-amino acid stretches of hydrophobic residues [7]. These hydrophobic stretches, similar to transmembrane domains, if exposed could be involved in anchoring the enzyme to the membrane. Efforts are under way to test this model.

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