Högberg-Raibaud, A., & Goldberg, M. E. (1977b) *Proc. Natl. Acad. Sci. U.S.A. 74*, 442–446.
Houmard, J., & Drapeau, G. R. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3506–3509.
Hummel, J. P., & Dreyer, W. J. (1962) *Biochim. Biophys. Acta 63*, 530–532.
Hyde, C. C., Ahmed, S. A., Padlan, E. A., Miles, E. W., & Davies, D. R. (1988) *J. Biol. Chem. 263*, 17857–17871.
Kirschner, K., Wiskocil, R. L., Foehn, M., & Rezeau, L. (1975) *Eur. J. Biochem. 60*, 513–523.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lane, A. N., & Kirschner, K. (1981) *Eur. J. Biochem. 120*, 379–387.
Lane, A. N., Paul, C. H., & Kirschner, K. (1984) *EMBO J. 3*, 279–287.
Miles, E. W. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 49*, 127–186.
Miles, E. W., & Moriguchi, M. (1977) *J. Biol. Chem. 252*, 6594–6599.
Miles, E. W., & Higgins, W. (1978) *J. Biol. Chem. 253*, 6266–6269.
Miles, E. W., Bauerle, R., & Ahmed, S. A. (1987) *Methods Enzymol. 142*, 398–414.
Revzin, A., & von Hippel, P. H. (1977) *Biochemistry 16*, 4769–4776.
Revzin, A. R., & Woychik, R. P. (1981) *Biochemistry 20*, 250–256.
Schnackerz, K. D. (1984) in *Chemical and Biological Aspects of Vitamin B_6 Catalysis: Part A* (Evangelopoulos, A. E., Ed.) pp 195–208, Alan R. Liss, Inc., New York.
Schnackerz, K. D., & Bartholmes, P. (1983) *Biochem. Biophys. Res. Commun. 111*, 817–823.
Seifert, T., Bartholmes, P., & Jaenicke, R. (1984) *Z. Naturforsch. 39C*, 1008–1011.
Seifert, T., Bartholmes, P., & Jaenicke, R. (1985) *Biochemistry 24*, 339–345.
Weischet, W. O., & Kirschner, K. (1976) *Eur. J. Biochem. 64*, 313–320.
Wiesinger, H., Bartholmes, P., & Hinz, H.-J. (1979) *Biochemistry 18*, 1979–1984.
Yanofsky, C., & Crawford, I. P. (1972) *Enzymes (3rd Ed.) 7*, 1–31.

Temperature and pH Dependence of the Self-Association of Human Spectrin†

G. B. Ralston
Department of Biochemistry, University of Sydney, Sydney 2006, Australia
Received July 30, 1990; Revised Manuscript Received November 30, 1990

ABSTRACT: The self-association of human spectrin between 21 and 35 °C and between pH 6.5 and 9.5 has been studied at sedimentation equilibrium. For a given set of solution conditions between pH 6.5 and 8.5, coincidence of Ω function plots as a function of total spectrin concentration (0–2 g/L) indicated that equilibrium was attained and that no significant concentration of solute was incapable of participating in the self-association reaction. Above pH 8.5, however, irreversible aggregation occurred, inferred from a failure of overlap in the Ω function and molecular weight distributions. The behavior of spectrin can best be described by a cooperative isodesmic model, in which the protomer for association is the heterodimer and for which K_{12} is between 10^6 and 10^7 M^{-1} (depending on pH and temperature) and all other K are approximately 10^6 M^{-1}. The returned values of the second virial coefficient for this model fall within the range calculated from the charge and Stokes radius of spectrin. Association appears to be favored slightly by decreased temperature and by decreased pH. The pH dependence resides only in K_{12} and is consistent with the presence of a single group, possibly histidine, displaying a slightly higher pK_a value in the tetramer than in the dimer. The association reaction appears to be driven by the loss of enthalpy associated with release of strain in the heterodimer. The association sites appear to be conserved in the association reactions, consistent with the images from electron microscopy. Within the precision of the data, the loss of rotational and conformational entropy on closing the oligomers from their open-chain forms is independent of the size of the oligomer.

Spectrin is the major protein of the erythrocyte membrane cytoskeleton that lines the cytoplasmic face of the red-cell membrane (Palek & Lux, 1983). The basic structural unit of spectrin is the heterodimer: a long, wormlike molecule consisting of two different polypeptide chains loosely wound around each other (Shotton et al., 1979). Spectrin is capable of self-associating through the sequential addition of heterodimers to form tetramers (Ralston, 1978; Shotton et al., 1979) and higher oligomers (Morrow & Marchesi, 1981; Morrow et al., 1981; Morris & Ralston, 1984; Liu et al., 1984).

In previous sedimentation equilibrium studies with spectrin at pH 7.5 and 30 °C (Morris & Ralston, 1989), it was not possible to decide unambiguously between two plausible models for indefinite self-association. In the "cooperative isodesmic" model (SEK III; Tang et al., 1977), the equilibrium constant in the molar scale for dimerization of the protomer, K_{12}, has a value different from the equilibrium constant in the molar scale that describes all subsequent additions of protomer to preexisting oligomers, K_{iso}. In the "attenuated indefinite" model (AK I; Adams et al., 1978), the sequential equilibrium constants are related to an "intrinsic constant", K, by the relationship $K_{i-1,i} = K/i$.

† This work was supported by the Australian Research Council.

0006-2960/91/0430-4179$02.50/0

The present study was performed over a range of solution pH and temperature in an attempt to resolve the ambiguity in reaction model and to explore the thermodynamic properties of the reaction.

Materials and Methods

Preparation of Spectrin. Packed human red cells, prepared from blood drawn from normal, healthy donors, were obtained from the Red Cross Transfusion Service, Sydney, Australia, and from the Seattle Blood Bank, Seattle, Washington, and were used within 48 h of collection. Spectrin heterodimer was extracted from the cells as previously described (Morris & Ralston, 1989) and was purified by repeated chromatography on a column of Sepharose CL-4B (Pharmacia) (3.0 × 50 cm) in a buffer comprised of 0.1 M NaCl/0.01 M sodium phosphate (pH 7.5)/5 mM EDTA/0.1 mM dithiothreitol/0.3 mM sodium azide/0.05 mM phenylmethanesulfonyl fluoride. After rechromatography, the central (2.5-mL) fraction from the center of the heterodimer peak was used immediately in order to minimize proteolytic damage.

For work over the range of pH from 6.1 to 9.5, the following buffers were used in overlapping pH regions: pH 6.1–7.1, 2-(*N*-morpholino)ethanesulfonic acid (MES); pH 6.8–8.0, 3-(*N*-morpholino)propanesulfonic acid (MOPS); pH 7.4–8.4, tris(hydroxymethyl)aminomethane (Tris); pH 7.5–7.8, sodium phosphate; pH 8.0–9.0, glycylglycine; pH 9.0–9.5, glycine. All buffers were made to be 10 mM in the ionized species, with the concentration of the free base or free acid adjustable. No consistent effect of the type of buffer on the parameters of self-association was detected. The working buffer solutions also contained 0.1 M NaCl, 5 mM EDTA, 0.1 mM dithiothreitol, 0.3 mM sodium azide, and 0.05 mM phenylmethanesulfonyl fluoride. The pH of the solutions was measured at 20 °C with the aid of a glass electrode, and the pH value at the temperature of interest was determined from the enthalpies of ionization of the buffers.

The central fraction from the dimer peak of rechromatographed spectrin either was used directly or was dialyzed overnight against the appropriate buffer. An aliquot of the diffusate was used as the reference buffer in sedimentation equilibrium experiments. In some experiments the spectrin fraction was concentrated by dialysis against dry Sephadex G-75 prior to dialysis against the relevant buffer. In the case of material taken directly from the column, the column buffer was taken as the reference buffer in dialysis equilibrium with the sample.

The pH range in these experiments was restricted to between 6.1 and 8.5. Below pH 6.1, spectrin aggregated and precipitated, while at pH values above 8.5 irreversible changes were found to occur. The accessible temperature range was between 21 and 35 °C. Below 21 °C, the rate of attainment of chemical equilibrium was impractically low, while problems of convection and temperature control were experienced above 35 °C. In experiments above 30 °C, 1% metrizamide was included in the buffers in order to stabilize against thermal convection (Ralston et al., 1989). No detectable difference in the association parameters due to the presence of the metrizamide was found at 30 °C, at which temperature some experiments were performed in both the presence and absence of metrizamide.

Meniscus Depletion Sedimentation Equilibrium. In the standard experiment, three different loading concentrations of spectrin (approximately 0.2, 0.5, and 1.0 g/L) were centrifuged at angular velocities of 6800 or 7200 rpm for up to 48 h in a Beckman–Spinco analytical ultracentrifuge fitted with electronic speed control and an RTIC unit. Sample volumes of 0.13 mL, giving a solution column of 3 mm, were routinely used. A titanium An-H rotor, a Yphantis 12-mm 6-channel centerpiece (Yphantis, 1964), and sapphire windows were used. The use of silicone layering oil was avoided in sedimentation equilibrium experiments (Morris & Ralston, 1984).

In order to check for attainment of equilibrium and the absence of contaminants, additional experiments at pH 7.5 and 30 °C were performed with initial loadings as low as 0.1 g/L and as high as 2.0 g/L, from the same preparation. Measurements were made at 6000 rpm with the lower sample loadings and up to 9000 rpm with the higher sample loadings; the higher angular velocities were required with the higher loadings in order to ensure meniscus depletion to within 0.1 fringe.

Routinely, a maximum concentration toward the cell bottom of 2–3 g/L was measurable. Numerical integration of the concentration distribution at equilibrium indicated that with the 0.2 g/L loadings at least 80% of the sample was detectable. With 0.1 g/L loadings, virtually all of the sample was visible.

For experiments over a range of temperatures, equilibrium was usually first attained at 35 °C, after which the temperature was lowered several degrees, and photographs were taken at the new temperature 4–16 h later. In some experiments, the temperature was raised from 21 °C to ensure that the results were independent of the direction of temperature adjustment.

At equilibrium, the Rayleigh interference pattern was recorded photographically on Kodak Spectroscopic IIG plates. The plates were measured on a Nikon comparator at 50× magnification, with the aid of an automated plate reader (DeRosier et al., 1972). A photograph was taken at 4000 rpm during acceleration for measurement of base-line correction (Teller, 1973). Since all experiments were carried out at an angular velocity of less than 10000 rpm, window distortion arising from high centrifugal fields during the run was not a major problem. A concentration conversion factor of 4.04 fringes per 1 g/L was used (Babul & Stellwagen, 1969).

The Ω function (Milthorpe et al., 1975) for a nonideal self-associating system is a continuous function of the total concentration of associating solute at any point in the centrifuge cell and of the parameters of the association reaction (the respective equilibrium constants and the second virial coefficient). If chemical equilibrium is attained, Ω function data calculated from the experimental data will superimpose on a single continuous curve, allowing both a test for the attainment of equilibrium (and the absence of contaminants) and an estimation of the parameters of self-association (Morris & Ralston, 1985).

To determine if all three spectrin samples in each experiment were homogeneous and had reached chemical equilibrium during the time course of the experiment, a reference concentration, $c(r_F)$, common to all three channels was chosen (usually 1.0 g/L) and the $\Omega(r)$ versus $c(r)$ curves were calculated from the experimental $c(r)$ data and examined for coincidence over their common concentration range

$$\Omega(r) = c(r) \exp[\phi_1 M_1(r_F^2 - r^2)]/c(r_F) \qquad (1)$$

where $\phi_1 = (1 - v\rho)\omega^2/2RT$, with v the partial specific volume of spectrin, ρ the solvent density, ω the angular velocity, R the universal gas constant, and T the absolute temperature. M_1, the protomer molecular weight for spectrin (i.e., the heterodimer), was taken as 480000 (Ralston, 1978), a value of 0.733 mL/g was used for the partial specific volume (Kam et al., 1977), and ρ was calculated to be 1.002 g/mL (Wolf et al., 1976). The square of the radial position r_F corresponding to

the reference concentration in each channel was estimated by interpolation with use of a six-point quadratic.

Data from initial loadings between 0.1 and 2.0 g/L from the same preparation at pH 7.5 and 30 °C showed excellent overlap of Ω function and $M_{w,app}$ distributions, indicating that all of the sample was capable of participating in the chemical equilibrium and that negligible contaminants were present. Furthermore, data from a wide range of experiments under these solution conditions, with samples from different preparations, all showed good overlap.

With other conditions of pH and temperature, data from all three channels in an experiment, as well as data obtained with different angular velocities, also usually showed good overlap. Data that did not display coincidence of Ω function plots over their common concentration range at sedimentation equilibrium were rejected.

The Adams–Fujita approximation (Adams & Fujita, 1963) for the thermodynamic activity of the protomer, $a_1(r)$, was used for all models

$$a_1(r) = c_1(r) \exp[BM_1c(r)] \quad (2)$$

where B, the second virial coefficient, is a measure of the nonideality of the solute, $c_1(r)$ is the promoter concentration, and $c(r)$ is the total spectrin concentration in grams per liter at radial distance r.

Model Fitting. The data were treated in several ways. First, from smoothed ln c versus r data (Teller, 1973), point-average weight-average molecular weight values were calculated and were used to compute the number and z average quantities. These quantities were used to determine sequential equilibrium constants and second virial coefficients for various nonideal discrete association schemes (Teller, 1973; Van Holde et al., 1969). Second, point-average weight-average molecular weight data were fitted with the SEK III and AK I self-association models (Morris & Ralston, 1984, 1989).

For the SEK III model (Tang et al., 1977), the monomer concentration, $c_1(r)$, is an implicit function of $c(r)$

$$c(r) = c_1(r)[1 + y(2 - x)/(1 - x)^2] \quad (3)$$

where $x = K_{iso}c_1(r)/M_1$ and $y = K_{12}c_1(r)/M_1$. The weight-average molecular weight is given by

$$M_w = 2yM_1/[1 + (1 - x)^3 + y(2 - x)(1 - x)] \quad (4)$$

For the AK I model (Adams et al., 1978)

$$c(r) = c_1(r) \exp[c_1(r)K/M_1] \quad (5)$$

$$M_w = M_1[1 + Kc_1(r)/M_1] \quad (6)$$

The apparent weight-average molecular weight in each case is given by

$$M_{w,app} = M_w/[1 + BM_wc(r)] \quad (7)$$

Finally, the various models were also fitted directly to the Ω function by means of nonlinear regression (Morris & Ralston, 1985, 1989):

$$\Omega(r) = a_1(r_F)c(r)/c(r_F)a_1(r) \quad (8)$$

In this equation, the $a(r)$ values were computed from eqs 3 or 5, in conjunction with eq 2.

The nonlinear regression program used for fitting weight-average molecular weight and Ω data was based on the Gauss–Newton algorithm. The reaction model parameters (equilibrium constant(s) and second virial coefficient) were reestimated for each iteration by use of the approximation by central differences of the partial first derivative of the fitting function (Cleland, 1967; Duggleby, 1984). Convergence to a final set of parameters was generally achieved for initial estimates on either side of the final values, with a tolerance of less than one part in 10^6. Approximate standard errors for the parameter estimates were calculated from the inverse matrix set up from the partial derivatives of the fitting function (Cleland, 1967) and were usually less than 15% of the parameter estimates themselves. In those cases where the standard errors exceeded 20% of the parameter estimates, the data were rejected. The Newton–Raphson iterative procedure was used to obtain $c_1(r)$ values when $c_1(r)$ was an implicit function of $c(r)$.

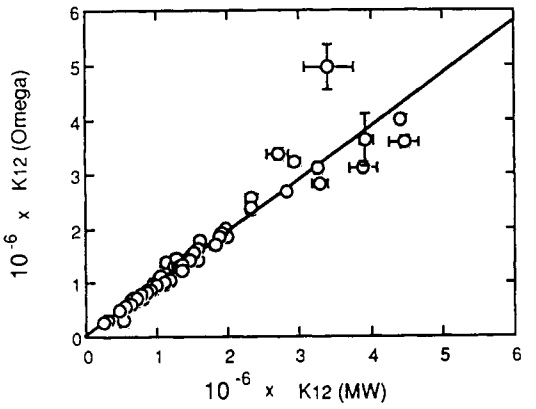

FIGURE 1: Correlation between K_{12} values for a SEK III model estimated from direct fitting of the Ω function and from fitting to the weight average molecular weight distribution. The error bars represent the approximate standard errors of the parameters (Cleland, 1967).

Purity of Spectrin. The purity of spectrin samples was examined by use of acrylamide gel electrophoresis in the presence of 0.2% SDS according to the method of Fairbanks et al. (1971). Heavy loadings were used to accentuate any impurities in the samples. Samples were found to be greater than 98% pure even at the completion of centrifugation. No traces of actin or band 4.1 could be detected in any of the samples used for sedimentation equilibrium experiments.

Results

Measurements made near 24 h after the start of ultracentrifugation frequently showed evidence of failure to reach equilibrium in the nonoverlapping of the weight-average molecular weight or Ω function plots for the three different loading concentrations. However, measurements at 36 h usually showed good overlap and did not change noticeably over the following 12 h except in the case of pH values above 8.5, under which conditions the Ω function and molecular weight plots diverged further with prolonged centrifugation, indicating irreversible reactions.

Method of Analysis. In the direct fitting of the Ω function for analysis of protein self-association, there exists the theoretical objection that the solute concentration appears in both ordinate and abscissa. In order to check the empirical validity of the Ω approach, the parameters of self-association determined from the Ω analysis were compared with those determined from the molecular weight distribution, both from the Dyson method (Van Holde et al., 1969) and from fitting of the models to the data through the use of nonlinear regression. Values of the parameter estimates from the entire range of experiments, covering the pH range 6.1–8.5 and the temperature range 21–35°, were examined.

As shown in Figure 1, there was excellent correlation between the parameters obtained from fitting the Ω function and those obtained from fitting the molecular weight distribution to a SEK III model. For the equilibrium constant for the dimer–tetramer step, K_{12}, a slope of 0.96 was determined, with a correlation coefficient of 0.96. The standard errors of the

parameter estimates obtained from the Ω analysis were routinely about half of those from the molecular weight distributions, a likely result of the increased uncertainty introduced into calculations of weight-average molecular weight through numerical differentiation.

Similarly, linear regression of the values of K_{iso} obtained from the Ω analysis on those from the molecular weight distribution showed a slope of 0.96 and a correlation coefficient of 0.97. The second virial coefficient also showed good correlation between estimates obtained from the Ω function and those obtained from the molecular weight distributions.

Estimates of K_{12}, determined from the Ω analysis with the SEK III model, correlated well with those obtained from the Dyson analysis of a nonideal heterodimer–tetramer–hexamer model. Here, the slope was 0.93 with a correlation coefficient of 0.89. However, substantially poorer correlation was found between the estimates for K_{23} from the Dyson procedure and K_{iso} from the fitting to the SEK III model either to the Ω data or to the molecular weight distribution (correlation coefficient = 0.68). This is not surprising, since the value of K_{23} from the Dyson approach was sensitive to the number of species used to model the data. With a heterodimer–tetramer–hexamer model, the fitting process always converged but values of the second virial coefficient were frequently negative, indicating that the degree of self-association had been underestimated. However, when the octamer was included in the model, convergence could not always be attained; in those cases that did converge, the value of K_{23} was smaller than in the case of the dimer–tetramer–hexamer model and was closer to the values of K_{iso} from the SEK III model.

The value of K_{12} estimated from the Dyson procedure, however, was relatively insensitive to the number of species used in the model, and thus the values obtained for this parameter are relatively model independent.

Choice of Model. Over the pH range 6.1–8.5 and over the entire temperature range used (21–35 °C), the self-association behavior of spectrin could be described adequately only by the cooperative indefinite model. All species participating in the reaction appeared to be in equilibrium, as judged by satisfactory overlap of both the Ω function versus concentration curves and the point-average weight-average molecular weight versus concentration curves. However, at pH 8.5, failure of overlap was apparent after 28 h and the curves diverged increasingly with time. At pH 9.5, the $M_{w,app}$ versus $c(r)$ curves were sigmoidal at all times and no overlap was attained.

At pH values above 7.0, it was not possible to distinguish unequivocally between the SEK III and AK I models on the basis of sums of squares of residuals or the distribution of residuals. Within experimental uncertainty, both models appeared to describe the behavior equally well, with similar values of the relevant equilibrium constants. Small differences in the value of the second virial coefficient appeared to be able to compensate for differences inherent in the two models, yet the differences in this parameter between the two models were within the uncertainty of its determination.

However, on the basis of the variation of the equilibrium constants with pH, the SEK III model seems to be the more appropriate. While in the AK I model all equilibrium constants are necessarily correlated, in the SEK III model K_{12} and K_{iso} are independent. Analysis of the pH dependence of K_{12} and K_{iso} from the fits to the SEK III model, from both $M_{w,app}$ and Ω function data, showed that while K_{12} was markedly pH dependent, K_{iso} was effectively independent of pH. When data for the entire range of conditions were examined, no correlation was found between K_{12} and K_{iso} or between K_{12} and K_{23}, as estimated from the Dyson procedure.

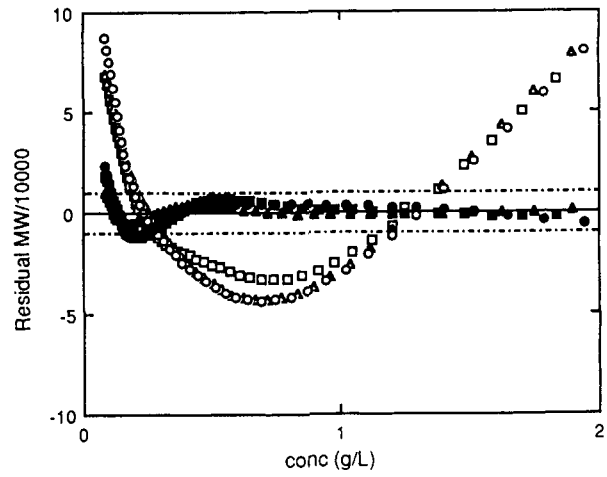

FIGURE 2: Plots of the residuals obtained by fitting the $M_{w,app}$ data for spectrin at 21 °C and pH 6.1 with (a) the SEK III model (filled symbols) and (b) the AK I model (open symbols). The data are from three separate channels with different loading concentrations. The broken lines represent the limits for 2% error in estimating the molecular weight of the heterodimer (480000). Except at concentrations below 0.2 g/L, the residuals from the SEK III fit lie wholly within this envelope.

Furthermore, at low temperatures and low pH values (conditions that favored the self-association reactions), the fit to a SEK III model was decidedly better than that to the AK I model for both the Ω function and the $M_{w,app}$ data (Figure 2). The residuals for the AK I fit under these conditions were markedly nonrandom and larger than experimental error. Finally, while the value of the second virial coefficient obtained from fitting the SEK III model increased slightly with increasing pH as it should, reflecting the increasing negative charge on the molecules, the value of B returned from fitting the AK I model decreased slightly with increasing pH and is thus physically implausible. Consequently, all subsequent analysis of the data was made in terms of the SEK III model.

Although some correlation of residuals for the SEK III model below 0.3 g/L may indicate a failure of the strict SEK III relationship, similar correlation of residuals was seen for all models examined and is more likely to reflect some convective erosion of the rather shallow concentration distribution in the centripetal part of the cell (D. C. Teller, personal communication).

pH Dependence. The pH dependence of K_{12} (Figure 3a) was analyzed according to the theory of linked functions (Wyman, 1964) in terms of a model in which a prototropic group or several groups on spectrin display different pK_a values in the heterodimer and tetramer, respectively. At constant temperature and pressure, and making the reasonable assumptions that the thermodynamic activity of solvent water and the binding of other solute species is independent of pH between pH 6.1 and 8.5, the pH dependence of K_{12} can be expressed as (Aune & Timasheff, 1971)

$$\text{d log } K_{12}/\text{d log } a_{H^+} = \Delta v_{H^+} = (v_{H^+})_{tetramer} - (v_{H^+})_{dimer} \quad (9)$$

where Δv_{H^+} is the difference between the numbers of protons bound to the two states in equilibrium. The maximum value of d log K_{12}/d log a_{H^+} was 0.56. Since this represents the lower limit of the number of association-linked prototropic groups, it is likely that only a single group in each heterodimer is implicated. According to this analysis, the data could be described adequately by a single group, presumably histidine, with a pK_a value of 6.79 in the heterodimer and a value of 7.99 in the tetramer. If two identical groups per heterodimer were

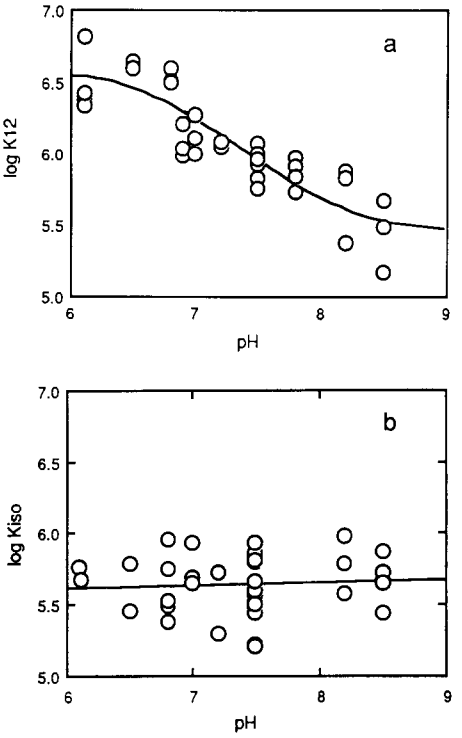

FIGURE 3: pH dependence of (a) log K_{12} and (b) log K_{iso} estimated from fitting the Ω function data from spectrin at 30 °C with a SEK III model. The solid line in (a) represents the best fit to a linked functions model in which a single prototropic group in the heterodimer of spectrin titrates with a pK_a of 6.79 in the heterodimer and with a pK_a of 7.99 in the tetramer. The solid line in (b) is the linear regression straight line of best fit to the data. The slope of this line is not significantly different from zero.

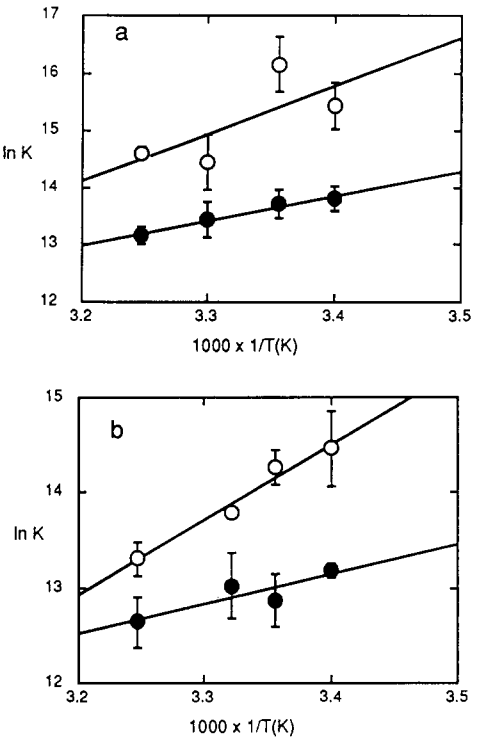

FIGURE 4: van't Hoff plots for K_{12} (open symbols) and for K_{iso} (closed symbols) for spectrin self association at (a) pH 6.1 and (b) pH 7.8. The points are the means of the values from the three individual channels, and the error bars show the standard deviation. The lines are the straight lines of best fit by unweighted linear regression. Within the limits of precision of the data, there appears to be no significant curvature of these plots over the accessible temperature range.

involved, the relevant pK_a values would be 7.06 in the dimer and 7.63 in the tetramer; the quality of fit to the data was indistinguishable for the two cases.

Both protonated and unprotonated forms of the dimer are capable of self-association, with the association of the protonated form being the stronger. It is interesting to note that the limiting value of K_{12} as the pH is raised is very close to the value of K_{iso} found throughout the pH range used in the present study.

The values of K_{iso} from analysis in terms of the SEK III model and the values of K_{23} from the Dyson analysis showed no significant pH dependence (Figure 3b).

Temperature Dependence. Over the range 21–35 °C, the values of the various equilibrium constants decreased with increasing temperature. Values of ln K were plotted against the reciprocal of the absolute temperature for data at pH 6.1 and 7.8 (Figure 4). At both pH values, the standard enthalpy change so obtained was negative for both the heterodimer–tetramer step and the subsequent steps (Table I). The slopes of the van't Hoff plots decreased slightly with increasing pH. However, there was no indication of curvature in the plots, within the limits of experimental uncertainty, from which it follows that ΔC_p is near zero for all steps of the self-association. The precision of these estimates of $\Delta H°$, on the basis of the standard errors of the slopes from the linear regression, is about 15–20%.

From the relationship

$$\Delta G° = \Delta H° - T\Delta S° \quad (10)$$

the standard entropy change was determined. The unitary entropy changes (Gurney, 1953) were calculated from the relationship

$$\Delta S_u = \Delta S° + R \ln 55.5 \quad (11)$$

Table I: Thermodynamic Parameters for the Self-Association of Human Spectrin at 30 °C[a]

conditions	$\Delta G°$ (kJ/mol)	$\Delta H°$ (kJ/mol)	$\Delta S°$ (J/mol·K)	ΔS_u[b] (J/mol·K)
		pH 6.1		
K_{12}	−38	−82	−144	−111
K_{23}	−33	−32	+3	+36
		pH 7.8		
K_{12}	−35	−65	−97	−64
K_{23}	−32	−25	+23	+56

[a] Solution conditions were 0.1 M NaCl, 0.01 M uni-univalent buffer ions, 5 mM disodium EDTA, 0.1 mM dithiothreitol, 0.3 mM sodium azide, and 0.02 mM PMSF. Thermodynamic functions were computed for 30 °C. The values of $\Delta H°$ and $\Delta S°$ were constant, within experimental uncertainty, over the range 21–35 °C. The uncertainty in the values of $\Delta G°$ is approximately ±2% and that in the enthalpy values is approximately ±20%, while that in the entropy may be higher. [b] The unitary change in entropy was calculated by adding R ln 55.5 to the standard change in entropy (Gurney, 1953).

and are also tabulated in Table I.

Plots of $\Delta G°$ versus absolute temperature were linear, and their slopes yielded direct estimates of the standard entropy change in good agreement with those obtained from $\Delta G°$ and $\Delta H°$, confirming the independence of $\Delta H°$ with temperature.

Discussion

Choice of Model. The self-association of spectrin can be considered in terms of an open form of the heterodimer, capable of self-associating in an isodesmic reaction through a series of "open-chain" forms (Figure 5). The association reactions involve complementary surfaces on each of the two polypeptide chains. The unsatisfied valencies at each end of these open-chain forms are considered capable of interacting through internal association to yield a series of "closed-ring" forms. Three relatively simple models are consistent with this

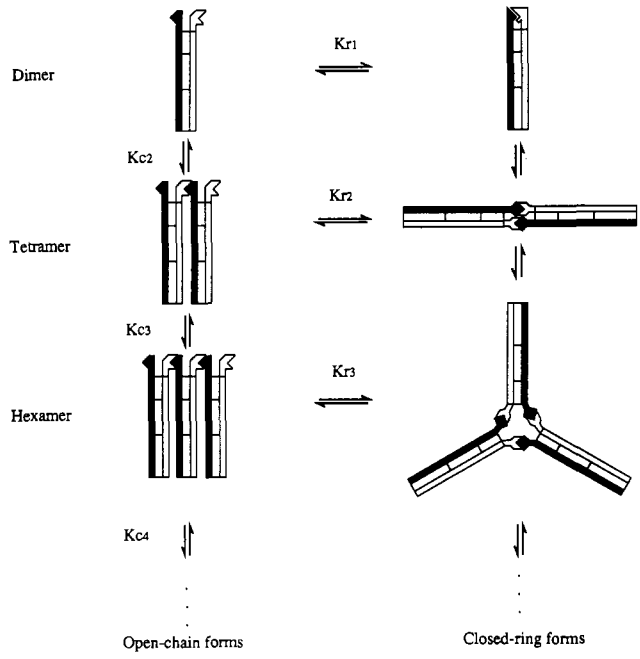

FIGURE 5: Reaction scheme for the self-association of spectrin. The K_{ci} values represent the association constant for addition of an open heterodimer to an open $(i - 1)$-mer to form an open i-mer of heterodimer units. The K_{ri} values are the equilibrium constants describing the formation of the closed ring forms from the open chain forms of the oligomer containing i heterodimer units.

scheme (Morris & Ralston, 1989): isodesmic, SEK III, and AK I. The three models are distinguishable by the relative values of the equilibrium constants for closing open-chain forms to produce the closed-ring forms (Morris & Ralston, 1989). The isodesmic scheme was rejected on the basis of nonrandom residuals and significantly greater standard deviation of the fits (Morris & Ralston, 1989).

At pH values above pH 7.0, it was not possible to discriminate unequivocally between the SEK III and AK I models on the basis of their ability to fit the experimental data from individual experiments. However, the SEK III model seems better able to describe the self-association behavior of spectrin over the entire set of solution conditions. Under conditions that favored association (low temperature and low pH), the SEK III model produced a significantly better fit to the data. In addition, over the entire range of conditions, K_{12} correlated poorly with K_{iso}; this poor correlation is evidence that the AK I model is inappropriate, since each of the individual equilibrium constants in this model is related to the "intrinsic" constant. Further grounds for rejecting the AK I model came from the physically implausible decrease in the second virial coefficient with increasing pH that was associated with this model.

Morris and Ralston (1989) showed that if self-association of spectrin proceeded through intermediate open-chain forms, expressions for the observed equilibrium constants could be derived

$$K_{12} = K_{c2}(1 + K_{r2})/(1 + K_{r1})^2 \quad (12)$$

$$K_{23} = K_{c3}(1 + K_{r3})/[(1 + K_{r1})(1 + K_{r2})] \quad (13)$$

where K_{ci} is the equilibrium constant for adding an additional open protomer to a preexisting open chain to form an oligomer of i protomers, K_{r1} is the equilibrium constant for closing the open heterodimer, K_{r2} is that for closing the tetramer, and K_{r3} is that for closing the hexamer (Figure 5).

If $K_{r1} < K_{r2} = K_{r3} = \ldots = K_r$ and if $K_{ci} = K_c$ for all i, then a SEK III mechanism would result (Morris & Ralston, 1989). From this mechanism, the following simplified expressions hold for K_{12} and K_{iso}:

$$K_{iso} = K_c/(1 + K_{r1}) \approx K_c/K_{r1} \quad (14)$$

$$K_{12} = K_c(1 + K_r)/(1 + K_{r1})^2 \approx K_cK_r/(K_{r1})^2 = K_{iso}(1 + K_r)/(1 + K_{r1}) \quad (15)$$

In the light of current data, K_{r1} has a value approximately half that of K_r at pH 7.5 and 30 °C (Morris & Ralston, 1989).

Thermodynamics of Spectrin Self-Association. All steps in the reaction were favored by decreasing temperature between 35 and 21 °C; the standard enthalpy change for all steps was negative. The magnitude of the loss in standard enthalpy decreased slightly with increasing pH for both the heterodimer–tetramer step and the subsequent steps of self-association. The standard enthalpy decrease for the isodesmic steps was approximately half the value for the heterodimer–tetramer step. Note, however, that from eqs 12–15, the calculated values of the thermodynamic functions $\Delta G°$, $\Delta H°$, and $\Delta S°$ refer only approximately to the closed heterodimer–closed tetramer reaction, to the extent that K_r and K_{r1} are much greater than unity.

The constancy of $\Delta H°$ over the accessible temperature range implies that the change in heat capacity for the self-association reaction is negligible. Since both hydrogen bonding and hydrophobic interactions are accompanied by substantial changes in heat capacity, the association reaction thus appears to conserve interactions between the subunits. That is, hydrophobic interactions and hydrogen bonding between the α and β chains in the heterodimer appear to be reformed between reciprocal α and β chains of the higher oligomers, as was originally suggested on the basis of electron microscopy (Shotton et al., 1979).

The measured change in the unitary entropy for the dimer–tetramer step in the reaction is negative and large. The change in entropy opposes the association reaction, which must therefore be enthalpy-driven. The unitary entropy changes estimated for the association reactions are relatively insensitive to changes in temperature, consistent with the negligible change in heat capacity. Since ΔC_p is effectively zero, major contributions to the unitary entropy change from changes in vibrational modes (Sturtevant, 1977) seem to be excluded. The remaining source of the relatively large and negative unitary entropy change is the loss of rotational modes or segmental motion in forming the tetramer. Although it is difficult to make a precise estimate for changes in the rotational entropy in the case of end-to-end association of a large, anisotropic molecule such as spectrin, the loss of rotational entropy in this step has been estimated to be 180 J/mol·K, on the basis of rigid spheres with a radius of gyration for the dimer of 22 nm (Elgsaeter, 1978) and for the tetramer of 31 nm. The calculated entropy change was insensitive to the actual value of the tetramer radius of gyration, varying only 5 J/mol·K for values between 22 and 44 nm.

Changes in conformational entropy may partly offset this entropy decrease: the tetramer and higher oligomers of spectrin seem to show partial unwinding of the two polypeptide chains with consequent increase in conformational flexibility of the molecule, compared with the dimer, in which the two chains are fairly closely apposed along their entire length. The tabulated value for the unitary change in entropy for the dimer–tetramer step thus appears to be reasonable.

The unitary entropy changes for the isodesmic steps in the reaction, however, are positive, though small in magnitude. Given that the SEK III model is appropriate for the self-association of spectrin, the surprising conclusion follows that,

Table II: Relative Thermodynamic Parameters for Closing of Open Oligomers of Spectrin[a]

pH	$\Delta\Delta G°$ (kJ/mol)	$\Delta\Delta H°$ (kJ/mol)	$\Delta\Delta S_u$ (J/mol·K)
6.1	−5	−50	−147
7.8	−3	−40	−120

[a] The values in this table represent the difference between the standard change for closing an oligomer of tetramer or larger and that for closing an open heterodimer.

for each step beyond the tetramer, the change in unitary entropy is constant (within the limits of the model) and positive, while that for the dimer–tetramer step is large and negative. From eqs 14 and 15, it follows that $K_{12}/K_{iso} \approx K_r/K_{r1}$. Thus, $\Delta G°_{12} - \Delta G°_{iso}$ reflects the difference in standard Gibbs free energy change between closing the open tetramer (or higher oligomer) and closing the open heterodimer: closing the open tetramer is more favorable by 3–5 kJ/mol, depending on the pH (Table II). Similarly, the difference in standard enthalpy and entropy changes for the formation of tetramer and for the subsequent isodesmic reactions reflects the standard enthalpy and entropy differences for closing the open tetramer (and all other open oligomers) and open heterodimer, respectively, and these values are listed in Table II. Closing the open tetramer is favored enthalpically by 40–50 kJ/mol over the closing of the open dimer but is opposed by a loss of entropy 120–150 J/mol·K greater.

The driving force for the reaction appears to be the negative standard enthalpy change on association. Since interactions within the association site appear to be conserved, this enthalpy effect may arise in large part from steric strain (Senear & Teller, 1981) in the closed heterodimer, arising from the different geometry of the interface in the dimer compared with that in the tetramer and higher oligomers. In this view, strain within the heterodimer appears to destabilize it by approximately 40 kJ/mol at pH 6.1 and 30 kJ/mol at pH 7.8. In forming the tetramer, the strain is released in two dimers, leading to a decrease in enthalpy of 80 kJ/mol at pH 6.1. In forming the hexamer from tetramer and dimer, the strain is released in a single additional dimer; thus the loss in enthalpy for the dimer–tetramer step is approximately twice that of the tetramer–hexamer step and subsequent steps.

The entropy difference between closing the tetramer and closing the dimer reflects the fact that, in closing the tetramer, the two component dimers lose much freedom of independent movement; when the dimer closes, the component polypeptide chains are already highly correlated, so no further large loss of rotational entropy can ensue. To the extent that a SEK III model is a good description of the reaction, the entropy of closing the open oligomers is independent of ring size for tetramer and higher oligomers.

Even if the SEK III model is found to be unrealistic, values of K_{23} obtained by the Dyson method, and also from application of the AK I model, are still quite close to those obtained from the SEK III model (Morris & Ralston, 1989), so the estimated entropies listed in Table I are relatively model independent for the tetramer–hexamer step and are not artifacts brought about by forcing an inappropriate model to fit the data.

pH Dependence. The pH dependence was not marked. The dimer–tetramer association step is linked to the net association of approximately half of a proton per dimer over the range pH 6.1 to pH 8.0. The apparent pK_a of the group involved suggests a histidine residue. A possible role for such a histidine could be in the formation of an electrostatic interaction with a carboxylic group, which would raise the apparent pK_a of the histidine from the near normal value in the dimer to the somewhat elevated value seen in the tetramer. This interaction is likely to contribute a small amount to the enthalpy of association at pH 6.1 compared with pH 7.8, consistent with the data of Table I. The precision of the data does not warrant inclusion of long-range electrostatic effects on the apparent pK_a values.

It is intriguing that while K_{12} shows clear pH dependence, K_{iso} does not. Lack of pH dependence for K_{iso} implies that the putative association-linked histidine is not actually in the association interface, since that would lead to pH dependence of K_c and hence to all steps of the association reaction. An explanation resides in the mechanism for the SEK III scheme: the only term appearing in the expression for K_{12} and not in that for K_{iso} (eqs 14 and 15) is K_r. Any pH dependence in this term would lead to pH dependence in K_{12} but not in K_{iso}. Such pH dependence of K_r can be envisaged through ionic interactions that are formed only in the closed higher oligomers. These interactions could arise either between charged groups on adjacent α and β polypeptide chains, respectively, or, more likely, between groups in adjacent domains within one of the chains. Presumably, steric strain or geometric constraint prevents such an interaction within the heterodimer.

In such an electrostatic interaction, one would also expect to see changes in the pK_a of the relevant carboxylate(s). However, the pH range for such an effect (below pH 5.0) is inaccessible in our experiments because of the insolubility of spectrin.

Alternatively, the pH dependence may result not from a specific ion-pair interaction but from generalized charge repulsion between the polypeptide chains; at the lower pH values, closer to the isoelectric point, repulsion is minimized. On the other hand, a generalized electrostatic effect is hard to reconcile with the pH dependence only of K_{12}.

It is interesting to note that at pH >9.0 the extrapolated value of $\Delta G°$ for the heterodimer–tetramer step is about −32 kJ/mol; i.e., with increasing pH the values of K_{12} and K_{iso} converge, resulting in a self-association reaction that becomes strictly isodesmic.

The present study has shown that the self-association behavior of spectrin conforms, at least over a limited degree of association, to the SEK III or cooperative indefinite model. Whether there is attenuation of the equilibrium constant for formation of very high oligomers, as might be expected from entropic considerations, must await studies at higher protein concentrations. The present study confirms that spectrin associates with conservation of the self-association interface, i.e., that even in the protomer the heterodimer, the interface between the α and β polypeptides is closed, though significantly strained. The particular geometry of spectrin thus makes it an unusual example of a protein that undergoes approximately isodesmic self-association through a series of closed oligomers, unlike the paradigm of isodesmic association in which free binding sites for self-association are always open and available for further accretion of protomers.

Even though the reaction from dimer to tetramer is driven against entropy by the strain developed in the heterodimer, the interactions between the two chains in the interface must still be very strong. No unequivocal dissociation of the dimer to constituent polypeptide chains has been detected under physiological conditions, down to 10 μg/mL.

ACKNOWLEDGMENTS

This work is dedicated to the memory of Professor J. W. Williams, as an admittedly inadequate tribute to his past guidance and teaching. The experimental work was performed largely in the laboratory of David Teller at the University of

Washington. To Professor Teller I owe a debt of gratitude for his help and hospitality. I am grateful to Ms. Catriona Grant for valuable assistance with some of the data analysis, to Dr. Michael Morris for many helpful discussions, and to Ms. Liz Harry for her critical reading of the manuscript.

REFERENCES

Adams, E. T., & Fujita, H. (1963) in *Ultracentrifugal Analysis in Theory and Experiment* (Williams, J. W., Ed.) pp 119-129, Academic Press, New York.
Adams, E. T., Tang, L.-H., Sarquis, J. L., Barlow, G. H., & Norman, W. M. (1978) in *Physical Aspects of Protein Interactions* (Catsimpoolas, N., Ed.) pp 1-55, Elsevier/North Holland, Amsterdam.
Aune, K. C., & Timasheff, S. N. (1971) *Biochemistry 10*, 1609-1622.
Babul, J., & Stellwagen, E. (1969) *Anal. Biochem. 28*, 216-221.
Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol. 21*, 1-32.
DeRosier, D. J., Munk, P., & Cox, D. J. (1972) *Anal. Biochem. 50*, 139-153.
Duggleby, R. G. (1984) *Comput. Biol. Med. 14*, 447-455.
Elgsaeter, A. (1978) *Biochim. Biophys. Acta 536*, 235-244.
Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606-2617.
Gurney, R. W. (1953) *Ionic Processes in Solution*, pp 80-112, McGraw Hill, New York.
Kam, Z., Josephs, R., Eisenberg, H., & Gratzer, W. B. (1977) *Biochemistry 16*, 5568-5572.
Liu, S.-C., Windisch, P., Kim, S., & Palek, J. (1984) *Cell 27*, 587-594.
Milthorpe, B. K., Jeffrey, P. D., & Nichol, L. W. (1975) *Biophys. Chem. 3*, 169-176.
Morris, M., & Ralston, G. B. (1984) *Biochim. Biophys. Acta 788*, 132-137.
Morris, M., & Ralston, G. B. (1985) *Biophys. Chem. 23*, 49-61.
Morris, M., & Ralston, G. B. (1989) *Biochemistry 28*, 8561-8567.
Morrow, J. S., & Marchesi, V. T. (1981) *J. Cell. Biol. 88*, 463-468.
Morrow, J. S., Haigh, W. B., & Marchesi, V. T. (1981) *J. Supramol. Struct. 17*, 275-287.
Palek, J., & Lux, S. E. (1983) *Semin. Hematol. 20*, 184-224.
Ralston, G. B. (1978) *J. Supramol. Struct. 8*, 361-373.
Ralston, G. B., Teller, D. C., & Bukowski, T. (1989) *Anal. Biochem. 178*, 198-201.
Senear, D. F., & Teller, D. C. (1981) *Biochemistry 20*, 3076-3083.
Shotton, D. M., Burke, B. E., & Branton, D. (1979) *J. Mol. Biol. 131*, 303-329.
Sturtevant, J. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2236-2240.
Tang, L.-H., Powell, D. R., Escott, B. M., & Adams, E. T. (1977) *Biophys. Chem. 7*, 121-139.
Teller, D. C. (1973) *Methods Enzymol. 27*, 346-441.
Van Holde, K. E., Rossetti, G. P., & Dyson, R. D. (1969) *Ann. N.Y. Acad. Sci. 164*, 279-293.
Wolf, A. V., Brown, M. G., & Prentiss, P. G. (1976) in *CRC Handbook of Chemistry & Physics* (Weast, R. C., Ed.) 57th ed., pp D218-D267, CRC Press, Cleveland, OH.
Wyman, J. (1964) *Adv. Protein Chem. 19*, 223-286.
Yphantis, D. A. (1964) *Biochemistry 3*, 297-317.

Examination of Sodium/Glucose Cotransport by Using a Visible Glucose Analogue†

Brian E. Peerce
Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, Texas 77550
Received October 8, 1990; Revised Manuscript Received December 28, 1990

ABSTRACT: The glucose derivative, 2,2,6,6-tetramethylpiperidine-1-oxylglucose (TEMPO-glucose) was synthesized and examined for its ability to substitute for glucose as a substrate for the intestinal brush border membrane Na^+/glucose cotransporter. TEMPO-glucose inhibited Na^+-dependent phlorizin binding with an apparent K_I of 18 μM and Na^+-dependent glucose uptake with an apparent K_I of 70 μM. The transport competence of TEMPO-glucose was examined by using two measures of transport. The first involved comparing the reversial of trans Na^+ inhibition by D-glucose and TEMPO-glucose. The second directly examined Na^+-dependent TEMPO-glucose uptake by using TEMPO-glucose quenching of intervesicular fluorescein sulfonate fluorescence. Tryptophan fluorescence was sensitive to TEMPO-glucose in a Na^+-dependent, glucose-inhibitable manner. The bulk of these tryptophans appeared to be located in hydrophobic environments based on Cs^+-insensitivity. With the reconstituted cotransporter, TEMPO-glucose, and tryptophan quench reagents, the cotransporter was compared in three transport modes: zero trans uptake, zero trans uptake in the presence of a shunt of membrane potential, and substrate exchange. The results suggest that the cotransporter conformation varies depending on its mode of operation and that TEMPO-glucose may be a useful probe for localizing amino acid residues involved in glucose transport.

A number of amino acids have been identified as important for substrate transport by Na^+/glucose cotransporters on the basis of inhibition of Na^+-dependent glucose uptake by amino acid specific reagents. The amino acids identified include tyrosines (Peerce & Wright, 1985; Lin et al., 1982; Wright & Peerce, 1985), lysines (Weber & Semenza, 1983; Peerce & Wright, 1984; Fernandez et al., 1989), sulfydryls (Klip et

† These studies were supported by USPHS Grants DK 34807 and DK 39944.

0006-2960/91/0430-4186$02.50/0