Lysosomal sulphate transport is dependent upon sulphydryl groups

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Using thiol blocking agents, we examined the role of sulphydryl groups for function of the lysosomal sulphate transport system. Monothiol binding reagents, *p*-hydroxymercuribenzoic acid (*p*-HMB) and *p*-chloromercuribenzenesulphonic acid (*p*-CMBS), dithiol binding reagents such as CuCl₂, the alkylating agent, *N*-ethylmaleimide (NEM), and NADH all inhibited lysosomal sulphate transport. The inhibitory effects of NEM and Cu²⁺ were not additive, suggesting that they both act upon the same critical sulphydryl group(s). Unlike the case for NEM, the inhibitory effects of Cu²⁺ were reversed by the reducing agent, dithiothreitol. Exposure to NEM resulted in a seven-fold increase in *K_m* to 867 μM versus a control value of 126 μM and a modest decrease

in *V_{max}* to 99 pmol per unit β-hexosaminidase per 30 s versus a control value of 129 pmol per unit β-hexosaminidase per 30 s. Similar although somewhat less dramatic results were obtained using Cu²⁺ with an increase of *K_m* to 448 μM and a *V_{max}* of 77 pmol per unit β-hexosaminidase per 30 s. The sulphate transport activity of detergent solubilized lysosomal membranes could be bound to a *p*-chloromercuribenzoic acid (*p*-CMB)–Sephacrose sulphydryl affinity resin and eluted with mercapto-ethanol. Sulphydryl groups thus appear to play a role in sulphate transport through effects on substrate affinity. Sulphydryl-binding appears to be a strategy that may be useful for purification of the transporter.

INTRODUCTION

Sulphate produced by the degradation of complex molecules such as glycosaminoglycans exits lysosomes by a specific, carrier-mediated, pH dependent transport system that can be reconstituted in artificial proteoliposomes [1–3]. Indirect evidence suggests that this transporter has some structural and functional similarities to the erythrocyte Band 3 anion exchanger [4]. Both systems have common sensitivities to a variety of inhibitors, some of which modify either lysine or arginine [4]. These inhibitor studies revealed that most divalent cations have either little or a small stimulatory effect on lysosomal transport. In contrast cupric ion, which does not have a major effect on transport by the erythrocyte anion exchanger, is quite inhibitory for the lysosomal system. Cupric ion is known to interact with histidine and thiol groups, suggesting possible mechanisms of action.

The importance of thiol groups for the function of numerous proteins involved in transport processes has been well demonstrated. Thiol dependent transporters include the vacuolar ATPase [5], the Ca²⁺ pump ATPase [6], simple channels for water and urea [7,8], gated chloride dependent K⁺ channel flux [9], and a variety of mobile carriers for glucose [10,11], nucleosides [12], choline [13], phosphate [14–16], amino acids [17], fatty acids [18], anions [18,19] and cations [20]. However, in earlier studies using lysosomal membrane vesicles the sulphate transporter did not appear to be particularly dependent upon sulphydryl (thiol) groups as NEM had no effect on trans-stimulation of sulphate transport [1].

In the present work, both permeant and impermeant sulphydryl-binding reagents were examined for their effects on lysosomal sulphate transport. As suggested by the findings with NEM and cupric ion, unidirectional transport appears to be quite sensitive to sulphydryl modification with dramatic effects on the kinetic characteristics of transport.

MATERIALS AND METHODS

Materials

All chemicals were reagent grade products and were purchased from Sigma unless otherwise indicated. [³⁵S]Na₂SO₄ was purchased from ICN Biochemical, Scintiverse II from Fisher, Dulbecco's phosphate-buffered saline without calcium from Irvine Scientific, AgNO₃ from Mallinckrodt, and GFC/GFF glass fibre filters from Whatman. Female Sprague–Dawley rats weighing 190–250 g were purchased from Harlan.

Methods

Lysosomal purification

Animals received intravenous injection of 1000 units of heparin 15 min prior to killing with CO₂ narcosis. Livers were removed and flushed with 1 mM EDTA in ice cold saline to remove blood. The livers were then homogenized in 1 mM EDTA/0.25 M sucrose. Lysosomes were purified 60–90-fold by differential centrifugation followed by density gradient centrifugation using 45% isotonic Percoll [21]. The lysosomal gradient fraction was removed, diluted with 10 volumes of 0.25 M sucrose, 2.0 mM Hepes, pH 7.0, and the lysosomes collected by centrifugation for 10 min at 13000 *g*. The pellet was resuspended in 0.25 M sucrose with 2 mM Hepes, pH 7.0. Activity of β-hexosaminidase was measured by fluorometric assay [22] and was defined as 1 unit = 1 μmol of product formed per min at 37 °C.

Membrane vesicle formation

The lysosomal Percoll gradient fraction was incubated for 15 min at 25 °C in 0.25 M sucrose, 20 mM Hepes, pH 7.0, with 1 μg/ml pepstatin, 2 μg/ml leupeptin, 2 mg/ml BSA and 5 mM methi-

Abbreviations used: *p*-HMB, *p*-hydroxymercuribenzoic acid; *p*-CMBS, *p*-chloromercuribenzenesulphonic acid; NEM, *N*-ethylmaleimide; *p*-CMB, *p*-chloromercuribenzoic acid; DIDS, 4,4'-diisothiocyanato-2,2'-disulphonic acid stilbene.

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onine methyl ester. Spontaneously resealed lysosomal membrane vesicles were separated by centrifugation at 20000 *g* for 15 min on a second Percoll gradient of identical composition. The vesicles at the top of the gradient were collected, washed and resuspended in buffer as described for lysosomes [1].

Sulphate transport

The standard assay mixture contained 2 μ Ci of [35 S]Na₂SO₄, 0.25 M sucrose, 20 mM Mes adjusted with NaOH to pH 5.0, 100 μ M Na₂SO₄, and 15 μ l of lysosomes (approx. 40–80 μ g protein) in a final volume of 50 μ l. For most studies, uptake was initiated by the final addition of sulphate to lysosomes. For kinetic studies uptake was initiated by the addition of lysosomes to the assay cocktail containing sulphate. The assay mixture was incubated for a 1 min interval at 25 °C and uptake was stopped by the addition of 1 ml of ice cold Dulbecco's phosphate buffered saline without calcium. Lysosomes were collected by filtration through GF/C filters and washed with 10 ml of ice cold buffered saline. Filters were dried and subjected to scintillation counting. Values were expressed as pmol of sulphate per unit β -hexosaminidase per min. Lysosomal membrane vesicles were treated similarly, but were collected using GF/F filters. Values were expressed as pmol of sulphate per mg protein per min. for kinetic studies, sulphate uptake was determined over a 30 s interval.

Transport in proteoliposomes

Lysosomal membrane proteins were solubilized with 3% Triton X-100 and mixed with egg yolk lecithin as previously described [3,23]. Proteoliposomes were spontaneously formed after the mixture was passed over a 1.6 ml mini-column containing Biobeads SN2. Approx. 25 μ l of reconstituted proteoliposomes (20 μ g protein) were incubated for 2 min at 25 °C with 5 μ l of a buffer mixture containing 228 mM Mes free acid, 600 μ M Na₂SO₄ and 2 μ Ci [35 S]Na₂SO₄, pH 5.0. Transport was stopped by the addition of 20 μ l of ice cold 0.18 M sucrose, 20 mM NaOH and 200 μ M DIDS, which effectively raised the pH to 7.4. Proteoliposomes were collected by gel permeation chromatography using Sephadex G-50 mini-columns. The excluded fraction was assayed for sulphate content by scintillation counting. The protein content of detergent-containing fractions was determined spectrophotometrically using bicinchoninic acid as a detection reagent [24].

Sulphydryl affinity chromatography

p-CMB–Sephadex resin was synthesized as described [24]. Approx. 100 μ l of detergent extracted lysosomal membrane protein was mixed with 150 μ l of affinity resin for 15 min on ice. The supernatant (unbound fraction) was collected by centrifugation for 5 min at 8000 *g* at 4 °C in an Eppendorf micro-centrifuge. The resin was first washed with 1 ml of 20 mM Hepes, 1% Triton X-100 adjusted with 1 M NaOH to pH 7.0 and followed by 1 ml of 0.15 M NaCl. The resin was re-equilibrated with 1% Triton X-100/28.5 mM sodium citrate, pH 7.0. Bound proteins were eluted using 0.1 ml of 3% Triton X-100, 28.5 M sodium citrate, pH 7.0 and 0.1% β -mercaptoethanol. The original extract, unbound and eluted fractions were desalted using 1 ml Sephadex G-50 spin columns before reconstitution.

RESULTS

Since sulphydryl interaction was a possible mechanism for the previously observed inhibitory effects of cupric ion, a variety of sulphydryl-binding reagents were examined for their effects on

Table 1 Effect of sulphydryl binding reagents on lysosomal sulphate uptake

Lysosomes were incubated for 1 min at 25 °C in 50 μ l of 0.25 M sucrose, 20 mM Mes, pH 5.0, 100 μ M Na₂SO₄, and 2 μ Ci of [35 S]Na₂SO₄ in the presence of sulphydryl-binding reagents as indicated. Lysosomes were collected by filtration through GF/C glass fibre filters and washed as described under 'Materials and methods'. Values are means $\pm$ S.D. of triplicate measurements. The control values are 36 $\pm$ 5 pmol per unit β -hexosaminidase per 30 s.

Sulphydryl-binding reagents	Sulphate uptake (% of control)
Control	100
<i>p</i> -HMB (50 μ M)	8 $\pm$ 0
<i>p</i> -CMBS (50 μ M)	11 $\pm$ 1
CuCl ₂ (100 μ M)	21 $\pm$ 0
FeCl ₂ (100 μ M)	130 $\pm$ 6
CoCl ₂ (100 μ M)	132 $\pm$ 1
AgNO ₃ (100 μ M)	9 $\pm$ 1
NADH (1 mM)	45 $\pm$ 5

Table 2 Reversible and irreversible inhibitors of lysosomal sulphate uptake

Lysosomes were incubated for 1 min at 25 °C in 50 μ l of 0.25 M sucrose/20 mM Mes (pH 5.0) or 20 mM Hepes, pH 7.0, 100 μ M Na₂SO₄, and 2 μ Ci of [35 S]Na₂SO₄ with additions as indicated. NEM was always preincubated with lysosomes at 25 °C for 5 min before transport assay. Lysosomes were collected by filtration through GF/C glass fibre filters as described under 'Materials and methods'. Each experiment included three determinations. Values are means $\pm$ S.D.

	Sulphate uptake					
	pH 5			pH 7		
	(pmol per unit of β -hexosaminidase per min)	% of control	<i>n</i>	(pmol per unit of β -hexosaminidase per min)	% of control	<i>n</i>
Control	46 $\pm$ 31	100	5	4.1 $\pm$ 0.5	100	4
NEM (5 mM)	11 $\pm$ 6	27 $\pm$ 8	5	3.7 $\pm$ 0.7	90 $\pm$ 14	4
Cu ²⁺ (50 μ M)	14 $\pm$ 6	31 $\pm$ 10	4	2.9 $\pm$ 0.9	75 $\pm$ 18	2
DTT (1 mM)	61 $\pm$ 34	118 $\pm$ 14	4	5.2 $\pm$ 0.1	38 $\pm$ 11	2
NEM + DTT	17 $\pm$ 17	25 $\pm$ 12	2	3.1 $\pm$ 1.2	81 $\pm$ 26	2
Cu ²⁺ + DTT	82 $\pm$ 29	143 $\pm$ 41	2	3.9 $\pm$ 2.2	101 $\pm$ 51	2
NEM + Cu ²⁺	16 $\pm$ 13	29 $\pm$ 6	2	2.7 $\pm$ 1.5	68 $\pm$ 36	2

sulphate uptake by lysosomes. At the concentrations employed, these sulphydryl inhibitors did not have significant effects on lysosomal stability nor did their anionic component significantly compete for sulphate uptake. As shown in Table 1, the organic-mercurial compound, *p*-HMB, the impermeant *p*-CMBS, CuCl₂ and AgNO₃ were effective inhibitors of sulphate uptake at pH 5.0, while CoCl₂ and FeCl₂ had no significant inhibitory effects. NADH [26,27], which is reported to interact with sulphydryl groups under certain conditions, inhibited sulphate uptake by 50%. Cupric ion failed to inhibit transport in the presence of the reducing agent, dithiothreitol (Table 2). Exposure of lysosomes to NEM also inhibited sulphate uptake and as expected due to the covalent nature of the interaction between NEM and sulphydryl groups, this effect was not reversed by exposure to dithiothreitol. The effects of cupric ion and NEM were not additive consistent with action at the same sites. A modest stimulation of transport was observed when lysosomes

Figure 1 Effect of NEM or cupric ion on sulphate transport

(A) Intact lysosomes isolated from rats were preincubated for 5 min with various concentrations of NEM in isotonic sucrose buffered with 2 mM Hepes, pH 7.0, at 25 °C. Transport was determined using an assay mix of 0.25 M sucrose, 20 mM Mes, pH 5.0 containing 2 μCi [³⁵S]Na₂SO₄ and 100 μM Na₂SO₄ as described in the Materials and methods section. (B) Sulphate transport by lysosomal membrane vesicles was determined by incubating membrane vesicles for 1 min in isotonic sucrose/Mes buffer as described in (A) but containing various concentrations of CuCl₂ as indicated. At the end of incubation intact lysosomes or membrane vesicles were filtered through GF/C or GF/F glass fibre filters respectively. The filters were washed, dried and counted. Rates of sulphate uptake were expressed as pmol per unit of β-hexosaminidase per 30 s for intact lysosomes and as pmol per mg of protein per min for membrane vesicles. Values represent the average of triplicate determinations.

were incubated with dithiothreitol alone. The effects of cupric ion and NEM were negligible when uptake was measured at pH 7.0, not surprising in view of the fact that rate of transport is reduced by 90% under these conditions as compared to the normal, acidic assay conditions. Similar effects were observed with both lysosomes and lysosomal membrane vesicles (data not shown).

Figure 2 Thiol reagents and the kinetics of lysosomal sulphate uptake

Lysosomes isolated from control rats (○) were either preincubated with 5 mM NEM for 10 min at 25 °C (●) or briefly (less than 10 s) exposed to 100 μM CuCl₂ (▽) and then incubated for 30 s at 25 °C in 0.25 M sucrose, 20 mM Mes, pH 5.0, containing 2 μCi [³⁵S]Na₂SO₄ and various concentrations of Na₂SO₄, as indicated. After incubation, lysosomes were collected and analysed as previously described. Values represent the average of triplicate determinations from one experiment. (A) Michaelis-Menten plot of the initial rate of sulphate uptake, *V*, as a function of sulphate concentration (*S*). (B) Hanes plot of the same data. *K_m* values for sulphate uptake were control 126 μM (*V_{max}* 139 pmol per unit of β-hex per 30 s) NEM-treated, 869 μM (*V_{max}* 99 pmol per unit of β-hex per 30 s) and cupric ion-treated 503 μM (*V_{max}* 77 pmol per unit of β-hex per 30 s).

Further studies were performed to study the mechanisms of action of cupric ion, a reversible inhibitor of transport and NEM, an irreversible inhibitor of transport. Both inhibitors exhibited concentration dependent effects. At 100 μM substrate concentrations, NEM was maximally effective at a concentration of 500 μM (Figure 1A) for lysosomes, while cupric ion was maximally effective at a concentration of 100 μM (Figure 1B) for

Table 3 Effect of substrate on inhibition by sulphydryl reagents

Lysosomes were incubated for 30 s at 25 °C in 50 μ l of 0.25 M sucrose/20 mM Mes, pH 5.0/200 μ M Na₂SO₄ $\pm$ 0.1 mM NEM. In some cases, lysosomes were preincubated with NEM at 25 °C for 5 min or 10 s before addition of the remaining incubation mixture. Values are mean $\pm$ S.D. from triplicate measurements. Statistical analysis was performed by Student's *t* test.

Order of addition	Sulphate uptake (pmol per unit of β -hexosaminidase per 30 s)	% of control	<i>P</i>
Lysosomes, buffer, then SO ₄ ²⁻	75 $\pm$ 5	100	—
Lysosomes, NEM for 5 min, then SO ₄ ²⁻	19 $\pm$ 2	26 $\pm$ 3	< 0.001
Lysosomes, NEM for 10 s, then SO ₄ ²⁻	39 $\pm$ 9	52 $\pm$ 12	< 0.005
Lysosomes, NEM with SO ₄ ²⁻	90 $\pm$ 10	120 $\pm$ 14	N.S.

lysosomal membrane vesicles. Kinetic analyses of sulphate uptake were performed using both inhibitors and revealed that the major effect of sulphydryl interaction was to alter substrate affinity. The K_m for sulphate uptake by lysosomes pre-treated with NEM was increased approx. seven-fold (NEM treated: 867 μ M versus control: 126 μ M (Figure 2)). The V_{max} was only moderately changed (NEM treated: 99 pmol per unit β -hexosaminidase per 30 s) versus control: 129 pmol per unit β -hexosaminidase per 30 s). Similar results were obtained when transport was examined in the presence of cupric ion although the results were somewhat less dramatic with a K_m of 448 μ M and a V_{max} of 77 pmol per unit β -hexosaminidase per 30 s (average of two experiments). In different experiments, variable results were obtained at low inhibitor concentrations, suggesting possible non-linearity. However, this was not consistent. The inhibitory effects of NEM were almost immediate requiring no more than 10 s of incubation before the addition of substrate. However, when NEM was added concurrent with substrate, there was no inhibition of transport, suggesting a protective effect of substrate (Table 3). This protective effect was overcome by increasing the concentration of NEM from 100 μ M to 5 mM, which resulted in an approx. 70 % decrease in transport. Substrate protection was also observed using cupric ion with 74 % inhibition of transport when lysosomes were briefly exposed to cupric ion before the addition of substrate. There was no inhibition when sulphate and cupric ion were added together.

Since the lysosomal sulphate transporter appears to have accessible sulphydryl groups, it is possible that sulphydryl affinity chromatography represents a means for purification of lysosomal membrane proteins. Detergent solubilized membrane proteins were mixed with *p*-CMB-Sepharose which was then extensively washed and eluted with mercaptoethanol as in 'Methods'. The various fractions were assayed for transport activity using reconstitution in proteoliposomes. Approx. 80 % of transport activity bound to the resin and 40 % was recovered in the eluted fraction (results not shown). The specific activity of the eluate increased approx. four-fold from 25 to 111 pmol per mg protein per 2 min. As previously reported, there was no uptake exhibited by protein-free liposomes [3].

DISCUSSION

Our studies indicate that lysosomal sulphate transport is dependent upon sulphydryl groups, thus explaining and confirming our earlier, preliminary observation of the inhibitory effect of cupric ion on sulphate uptake. Mono-thiol binding reagents such

as *p*-HMB and *p*-CMBS as well as dithiol-binding reagents such as Cu²⁺ inhibit sulphate uptake by lysosomes. However, not all metal ions have this inhibitory effect. This may be explained by decreased affinity for thiol groups and formation of unstable products with Co²⁺ and Fe²⁺. The inhibitory effect is certainly not confined to metals, as transport is inhibited by diverse sulphydryl-binding agents such as the alkylating agent, NEM and NADH. As expected, the sulphydryl-reducing agent dithiothreitol protects against the inhibitory effects of reversible sulphydryl-binding compounds and by itself appears to stimulate sulphate uptake slightly. While it is likely that the lysosomal sulphate transporter contains more than one sulphydryl group, it is possible that only one or a limited number of sulphydryl groups is involved in the transport process. We found no evidence for stepwise inhibition of transport with increasing concentrations of NEM as is sometimes seen when a protein contains a number of sulphydryls of varying accessibility, all of which are important for function. However, the apparent non-linearity found in some experiments at low inhibitor concentrations may be evidence of involvement of two or more sulphydryl groups. The fact that the effects of cupric ion and NEM are not additive suggests that the sulphydryl group(s) that are involved in the transport process are equally accessible to these inhibitors.

Studies with permeant and impermeant sulphydryl-blocking reagents have been employed to determine the location of essential sulphydryl groups for choline transport [13], Band 3 anion transport [28] and glucose transport [29] by human red blood cells. For lysosomal sulphate transport, inhibition by impermeant compounds such as *p*-CMBS and NADH suggests that sulphydryl groups which are critical for function of the transporter are accessible from the external or cytoplasmic surface of the lysosome.

The fact that, under certain conditions, the presence of substrate can completely prevent the inhibitory effects of NEM or cupric ion is intriguing. This protective effect provides an explanation for the insensitivity of sulphate uptake to sulphydryl-binding by NEM in trans-stimulation experiments [1], since transport is measured in membrane vesicles that have been formed in the presence of both NEM and high concentrations of sulphate. The mechanism for the protective effect is uncertain, although there are a number of possible explanations. An essential sulphydryl may be located near or may even be a part of the substrate binding site. Occupation of the binding site with substrate would then block access to this critical sulphydryl group(s). Findings using impermeant inhibitors suggest that this sulphydryl is accessible from the external surface. This hypothesis would be consistent with the increased K_m observed after exposure of lysosomes to cupric ion or NEM. Sulphydryl-binding would presumably result in steric hindrance or direct alteration of the actual binding site with altered K_m and decreased transport. A second explanation for the protective effect of substrate does not require the key sulphydryl to be located near the substrate binding site. In this model, conformational changes induced by substrate binding would make key sulphydryls inaccessible to either NEM or cupric ion. In order to account for inhibition of transport, sulphydryl binding in the absence of substrate would induce conformational changes that limit accessibility to the substrate binding site.

Our studies have given us some insight regarding the structure and function of the lysosomal sulphate transporter. However, isolation of the protein and gene will be necessary for further understanding. The preliminary studies with affinity chromatography suggest that it may be possible to exploit the sulphydryl structure of the lysosomal sulphate transporter to achieve these aims through protein purification.

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REFERENCES

- 1 Jonas, A. J. and Jobe, H. (1990) *J. Biol. Chem.* **265**, 17545–17549
- 2 Chou, H. F., Passage, M. and Jonas, A. J. (1994) *J. Biol. Chem.* **269**, 23524–23529
- 3 Koettters, P., Chou, H. F. and Jonas, A. J. (1995) *Biochim. Biophys. Acta* **1244**, 311–316
- 4 Koettters, P., Chou, H. F. and Jonas, A. J. (1995) *Biochim. Biophys. Acta* **1235**, 79–84
- 5 Feng, Yu and Fargac, M. (1992) *J. Biol. Chem.* **267**, 5817–5822
- 6 Vincenzi, F. F. and Wu, L. (1988) *Proc. West. Pharmacol. Soc.* **31**, 231–233
- 7 Benga, G., Popescu, O. and Pop, V. I. (1986) *Biochemistry* **25**, 1535–1538
- 8 Toon, M. R. and Solomon, A. K. (1986) *Biochim. Biophys. Acta* **860**, 361–375
- 9 Smith, D. K. and Lauf, P. K. (1985) *Biochim. Biophys. Acta* **818**, 251–259
- 10 May, J. M. and Beechem, J. M. (1993) *Biochemistry* **32**, 2907–2915
- 11 Pinkofsky, H. B. and Jung, C. Y. (1985) *Arch. Biochem. Biophys.* **240**, 94–101
- 12 Tse, C. M., Wu, J. S. R. and Young, J. D. (1985) *Biochim. Biophys. Acta* **818**, 316–324
- 13 Krupa, R. M. and Deves, R. (1988) *J. Membrane Biol.* **101**, 43–47
- 14 Kaplan, R. S., Pratt, R. D. and Pedersen, P. L. (1986) *J. Biol. Chem.* **261**, 12767–12773
- 15 Pratt, R. D. and Pedersen, P. L. (1989) *Arch. Biochem. Biophys.* **268**, 9–19
- 16 Yamaguchi, T. and Kimoto, E. (1992) *Biochemistry* **31**, 1968–1973
- 17 Widmer, J., Gaillard, J. M., Bovier, P. and Tissot, R. (1989) *Neuropsychobiology* **21**, 53–58
- 18 Deuticke, B. (1986) *Membrane Biochem.* **6**, 309–326
- 19 Zhang, Z. H. and Solomon, A. K. (1992) *Biochim. Biophys. Acta* **1106**, 31–39
- 20 Shi, Z. Q., Davison, A. J. and Tibbits, G. F. (1989) *J. Mol. Cell Cardiol.* **21**, 1009–1016
- 21 Jonas, A. J. (1986) *Biochem. J.* **36**, 671–677
- 22 Shapira, G., Blitzer, M. G., Miller, J. B. and Africk, D. K. (1989) *Biochemical Genetics: A Laboratory Manual*, pp. 28–32, Oxford University Press, New York
- 23 Dawson, R. M. C. (1963) *Biochem. J.* **88**, 414–423
- 24 Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallio, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985) *Anal. Biochem.* **150**, 76–85
- 25 Casey, J., Lieberman, D. M. and Reithmeier, R. A. F. (1989) *Methods Enzymol.* **173**, 502–505
- 26 Jocelyn, P. C. (1972) in *Biochemistry of the SH Group*, pp. 74–190, Academic Press, New York
- 27 Watkins, D., Cooperstein, S. J. and Lazarow, A. (1971) *Endocrinology* **88**, 1380–1384
- 28 Werner, P. K., Lieberman, D. M. and Reithmeier, R. A. F. (1989) *Biochim. Biophys. Acta* **982**, 309–315
- 29 Abbott, R. E., Schachter, D., Batt, E. R. and Flamm, M. (1986) *Am. J. Physiol.* **250**, C853–C860

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