Infrared spectroscopy of chemically modified heparins

David GRANT, William F. LONG,* Colin F. MOFFAT and Frank B. WILLIAMSON

Department of Biochemistry, University of Aberdeen, Marischal College, Aberdeen AB9 1AS, Scotland, U.K.

Analysis by i.r. spectroscopy of natural and chemically modified heparins suggests that the *N*-sulphonate group of these polymers may vibrationally absorb radiation at two distinctly different frequencies. This property may reflect the existence of different conformational states important in the biological activities of the polymers. Examination of carboxylate- and *N*-acetyl-group absorbances of these polymers accords with the possibility that one of these polymer conformational states involves interaction between juxtaposed *N*-sulphonate and carboxylate groups. In heparin bearing large quantities of artificially introduced *N*-acetyl groups, an interaction between carboxylate and *N*-acetyl groups may also occur.

DISCUSSION

Heparins and heparans are glycosaminoglycans composed of substituted repeating disaccharide units of D-glucosamine and uronic acid residues. Heparins are widely used pharmacologically as anticoagulants, are components of mast cells, and are of uncertain physiological function. Heparans contain less sulphate groups and iduronic acid residues, and more acetyl groups and glucuronic acid residues, than heparins, are present in mammalian pericellular matrices, and apparently play roles in cell adhesion and in the determination of the proliferative state of cells.

The *N*-sulphonate group attached to many of the glucosamine residues of heparins and heparans is essential to a variety of the biological activities of the polymers. A recent analysis by wide-range i.r. spectroscopy of a series of heparin- and heparan-metal-cation complexes suggested that this group may absorb radiation at two distinctly different frequencies, including one at 1230–1235 cm⁻¹ not previously noted (Grant *et al.*, 1987a). The frequency at which the *N*-sulphonate absorbance predominantly occurs depends upon the identity of the polymer counteranion and the conditions of sample preparation. Such spectroscopic differences could reflect the existence of different conformational states, important in the biological activities of the polymers (Grant *et al.*, 1987b). In the present paper the results of i.r. spectroscopy of well-characterized chemically modified heparins are shown to accord with our previous analyses of *N*-sulphonate absorbance patterns.

EXPERIMENTAL

Pig intestinal-mucosal heparin [grade II; 167 USP (United States Pharmacopeia) units·mg⁻¹; batch 122F-0756] was from Sigma Chemical Co., St. Louis, MO, U.S.A. *N*-Desulphonation involved incubating the pyridinium salt of heparin in DMSO/water (19:1, v/v) for 1.5 h at 90 °C; partial (48 %) *N*-desulphonation involved similar incubation for 2 h at 20 °C (Inoue & Nagasawa, 1976). *N*-Desulphonation together with

O-desulphation involved incubation in DMSO/methanol (9:1, v/v) for 10 h at 100 °C (Nagasawa *et al.*, 1977; Nagasawa & Inoue, 1980). *N*-Resulphonation of *N*-desulphonated, *O*-desulphated heparin involved use of a pyridine-sulphur trioxide complex; sequential incubations led to polymers that were 54, 89 and 100 % *N*-resulphonated (Levy & Petracek, 1962; Lloyd *et al.*, 1964; Inoue & Nagasawa, 1973; Bruce *et al.*, 1985). Partially (83 %)-carboxy-reduced heparin was prepared by the method of Taylor & Conrad (1972). Further modifications involved production of *N*-desulphonated *N*-acetylated heparin and *N*-desulphonated *O*-desulphated *N*-acetylated heparin. The latter was prepared by two routes: (a) *N*-acetylation before *O*-desulphation, and (b) *N*-acetylation after *O*-desulphation. The *N*-acetylation was carried out with acetic anhydride by using the method of Nagasawa & Inoue (1980).

Polymers were characterized by uronic acid determination (Blumenkrantz & Asboe-Hansen, 1973), acid/base titration and ¹³C n.m.r. spectroscopy using a Brücker WH-360 spectrometer operating at 90.56 MHz with quadrature detection. Further details of polymer modification and characterization were given by Moffat (1987). I.r. spectroscopy of aqueous films of Na-heparin complexes was by a multiple-diffuse-reflectance technique (Grant *et al.*, 1987a).

RESULTS AND DISCUSSION

N-Sulphonate-group absorbances

I.r. spectra of native, partially *N*-desulphonated and *N*-desulphonated heparins are shown in Fig. 1(a), spectra a, b and c respectively; absorbances, with maxima at 1190 and 1230 cm⁻¹ and at 1190 cm⁻¹, attributable to the presence of *N*-sulphonate groups in the native polymer, are shown in the difference spectra Fig. 1(b), spectrum a [i.e. spectrum (a)c subtracted from (a)a] and spectrum b [i.e. (a)c subtracted from (a)b] respectively. Spectra of *N*-desulphonated *O*-desulphated heparin, and of samples of this polymer that had been *N*-resulphonated to various extents, are shown in Fig. 1(a), spectra d–g. Absorbances, with maxima at 1230 and shoulders at about 1185 cm⁻¹, attributable to the presence of *N*-sulphonate groups in

Abbreviations used: DMSO, dimethyl sulphoxide.

* To whom correspondence and reprint requests should be sent.

Fig. 1. I.r. spectra

(a) I.r. spectra, between 1100 and 1300 cm^{-1} , of native and chemically modified heparins: a, native heparin; b, partially *N*-desulphonated (48 %) heparin; c, *N*-desulphonated heparin; d, *N*-desulphonated, *O*-desulphated heparin; e, *N*-desulphonated, *O*-desulphated, *N*-resulphonated (54 %) heparin; f, *N*-desulphonated, *O*-desulphated, *N*-resulphonated (88 %) heparin; g, *N*-desulphonated, *O*-desulphated, *N*-resulphonated (100 %) heparin. Discrete *N*-sulphonate absorbances are indicated by arrows. (b) I.r. difference spectra derived from (a): a, spectrum (a)a minus (a)c; b, spectrum (a)b minus (a)c; c, spectrum (a)e minus spectrum (a)d; d, spectrum (a)f minus spectrum (a)d; e, spectrum (a)g minus spectrum (a)d. Discrete *N*-sulphonate absorbances are indicated by arrows. (c) I.r. spectra, between 1400 and 1500 cm^{-1} , of native and chemically modified heparin: a, native heparin; b, partially *N*-desulphonated (48 %) heparin; c, *N*-desulphonated heparin; d, *N*-desulphonated, *O*-desulphated heparin; e, *N*-desulphonated, *O*-desulphated, *N*-resulphonated (54 %) heparin; f, *N*-desulphonated, *O*-desulphated (88 %) heparin; g, *N*-desulphonated, *O*-desulphated, *N*-resulphonated (100 %) heparin; h, partially-carboxy-reduced (83 %) heparin; i, the difference spectrum (a minus h). (d) I.r. spectra, between 1300 and 1400 cm^{-1} and between 1500 and 1650 cm^{-1} , of native and chemically modified heparins: a, native heparin; b, partially *N*-desulphonated (48 %) heparin; c, *N*-desulphonated heparin; d, *N*-desulphonated, *O*-desulphated heparin; e, *N*-desulphonated, *O*-desulphated, *N*-resulphonated (54 %) heparin; f, *N*-desulphonated, *O*-desulphated, *N*-resulphonated (88 %) heparin; g, *N*-desulphonated, *O*-desulphated, *N*-resulphonated (100 %) heparin; h, *N*-desulphonated, *O*-desulphated, *N*-acetylated heparin (*N*-acetylation before *O*-desulphation); i, *N*-desulphonated, *O*-desulphated, *N*-acetylated heparin (*N*-acetylation after *O*-desulphation); j, *N*-desulphonated, *N*-acetylated heparin. (e) I.r. difference spectra derived from (d): a, spectrum (d)h minus (d)d; b, spectrum (d)i minus spectrum (d)d; c, spectrum (d)j minus spectrum (d)c.

the *N*-resulphonated polymers, are shown in the difference spectra Fig. 1(b), spectra c-e [i.e. (a)d subtracted from (a)e-(a)g respectively].

An absorption band at 1185 cm^{-1} , occurring in $^2\text{H}_2\text{O}$ solutions of heparins, was previously attributed to the *N*-sulphonate groups of the polymer (Cabassi *et al.*, 1978). We, too, observed this absorbance, in spectra of $^2\text{H}_2\text{O}$ solutions of heparin, of dilute (less than 10 %, w/v) aqueous solutions of heparins and heparans at low ionic strength, and of complexes between heparin and quat-

ernary-amine salts (Grant *et al.*, 1987a). In contrast, spectra of hydrated and of totally or partially dehydrated heparin films, and of aqueous solutions of heparin at higher concentrations (greater than 10 %, w/v), showed no 1185 cm^{-1} *N*-sulphonate band. Spectra of aqueous solutions of heparans at concentrations greater than 10 % (w/v) similarly showed no 1185 cm^{-1} band, but contained a previously unreported band at 1230 cm^{-1} , eliminated by prior *N*-desulphonation of the polymer. It was suggested that, for heparins, *N*-sulphonate absorb-

ance at 1230 cm^{-1} is obscured by strong *O*-sulphate-group absorbance in this region, and that the *N*-sulphonate absorbance at 1230 cm^{-1} is only readily discernible in spectra of the more poorly *O*-sulphated heparans (Grant *et al.*, 1987a).

The present analysis of chemically modified heparins clearly accords with the notion that heparin *N*-sulphonate groups may absorb around $1230\text{--}1235\text{ cm}^{-1}$ as well as over the range $1180\text{--}1200\text{ cm}^{-1}$.

Carboxylate-group absorbances

The spectroscopic properties of the *N*-sulphonate group could reflect the existence of different polymer conformational states, important in the biological activities of the polymer (Grant *et al.*, 1987b). It has been suggested that one such conformation might involve hydrogen-bonding between the *N*-sulphonate groups and adjacent iduronate carboxylate groups (Grant *et al.*, 1987b), brought into juxtaposition by rotation around the glycosidic oxygen of about 180° from a previously described heparin conformation (Gatti *et al.*, 1979).

I.r. spectra, between 1400 and 1500 cm^{-1} , of native and of chemically modified heparins are shown in Fig. 1(c). In spectra of native heparin and of *N*-desulphonated *O*-desulphated preparations that had been *N*-resulphonated (to 100%), the carboxylate symmetric stretching absorbance occurred predominantly at $1429\text{--}1432\text{ cm}^{-1}$, with shoulders in the spectrum indicating additional minor absorbance at about $1415\text{--}1419\text{ cm}^{-1}$ (Fig. 1c, spectra a and g). Spectra of *N*-desulphonated, and of *N*-desulphonated *O*-desulphated, preparations showed a corresponding, predominant absorbance at about $1415\text{--}1419\text{ cm}^{-1}$ (Fig. 1c, spectra c and d). The spectrum of *N*-desulphonated *O*-desulphated heparin that had been *N*-resulphonated to 54% showed absorbance peaks at 1417 and 1433 cm^{-1} (Fig. 1c, spectrum e), and those of *N*-desulphonated (48%) heparin and *N*-desulphonated *O*-desulphated heparin that had been *N*-resulphonated (88%), absorbances with maxima at about 1424 cm^{-1} (Fig. 1c, spectra b and f). Spectrum h of Fig. 1(c) shows that of a polymer from which carboxy groups have been partially and selectively removed by reduction; the spectrum, when compared with that of native heparin, shows reduced absorbance between 1415 and 1435 cm^{-1} , in accord with our assignment of such absorbance in other polymers to the carboxy group. The difference spectrum shown in Fig. 1(c), spectrum i [spectrum (c)h subtracted from (c)a], with its predominant absorbance around 1430 cm^{-1} and shoulder at about 1417 cm^{-1} , accords with the assignment of similar absorbances, present in the spectrum of native heparin, to the carboxy group.

We tentatively interpret these results thus: the carboxylate absorbance may occur over at least two discrete frequency ranges, at about $1429\text{--}1432\text{ cm}^{-1}$ and at about $1415\text{--}1419\text{ cm}^{-1}$ respectively; there is a possibility of carboxylate absorbance at intermediate frequencies (Fig. 1c, spectrum b). Since absorbance at $1415\text{--}1419\text{ cm}^{-1}$ occurs in spectra of preparations lacking *N*-sulphonate groups, and since strong absorbance at $1429\text{--}1432\text{ cm}^{-1}$ occurs in preparations containing *N*-sulphonate groups absorbing predominantly at about 1220 cm^{-1} , we suggest that the $1429\text{--}1432\text{ cm}^{-1}$ absorbance (indicated by solid arrows in Fig. 1c) is that of carboxylate groups involved in interaction with adjacent *N*-sulphonate groups, whereas that at 1415--

1419 cm^{-1} (indicated by dotted-line arrows in Fig. 1c) is that of carboxylates not so interacting. Previous studies indicate that, in the presence of cations, poorly interacting with heparin carboxylates, the carboxylate absorbance is centred at 1415 cm^{-1} , whereas with increasing cation-carboxylate interaction, the absorbance moves to higher frequencies (Grant *et al.*, 1987a).

N-Acetyl amide-II and methyl C-H bending absorbances

The above results suggest the possible occurrence of an interaction between juxtaposed *N*-sulphonate and carboxylate groups of heparins. If this were so, then in circumstances in which such interaction is suggested to occur, and in circumstances in which such an interaction is made impossible by previous chemical removal of a putatively interacting group, we would expect to detect polymer conformational differences reflected in absorbances of groups other than those participating directly in the interaction. Because the *N*-acetyl amide-II absorbance of heparin is sensitive to counteraction identity (Fig. 1 in Grant *et al.*, 1987a), we examined the *N*-acetyl absorbance of native and chemically modified heparins.

I.r. spectra between 1300 and 1400 cm^{-1} and between 1500 and 1650 cm^{-1} , of native and of chemically modified heparins, are shown in Fig. 1(d), in which the absorbances discussed in the text are indicated by arrows. We ascribe bands at $1530\text{--}1565\text{ cm}^{-1}$ to amide-II vibrations of *N*-acetyl groups on the basis of their co-occurrence with the acetyl methyl group symmetrical C-H deformation bands known to occur at about 1380 cm^{-1} (She *et al.*, 1974), and the enhancement of absorbance around $1530\text{--}1565\text{ cm}^{-1}$ in heparins with increased *N*-acetyl content (Fig. 1d, spectra h, i and j, and Fig. 1e). Native heparin, which contains only a small number of *N*-acetyl groups, produces a spectrum with a shoulder at 1560 cm^{-1} (Fig. 1d, spectrum a), which becomes a more pronounced peak at 1530 cm^{-1} when the heparin is *N*-desulphonated (Fig. 1d, spectrum c). An intermediate condition is shown in Fig. 1(d), spectrum b.

The results accord with the idea that a polymer conformation form, stabilized by *N*-sulphonate-carboxylate interaction, is destabilized after removal of *N*-sulphonate groups. The absence of a shift in the iduronate 2-*O*-sulphate C—O—S bending vibration absorption frequency from 800 cm^{-1} (equatorial) to 860 cm^{-1} (axial) upon *N*-desulphonation suggests that a $^1\text{C}_4 \rightarrow ^4\text{C}_1$ flipping of the iduronate ring is not responsible for the altered amide-II absorbance. Upon chemical reduction of iduronate 2-*O*-sulphate to idose 2-*O*-sulphate, in which a $^1\text{C}_4 \rightarrow ^4\text{C}_1$ conformational change is likely, such a C—O—S change does occur (results not shown). Strong, but poorly resolved, amide-II bands at low frequency ($1530\text{--}1549\text{ cm}^{-1}$) are observed for *N*-desulphonated *O*-desulphated heparin (Fig. 1d, spectrum d), and progressive *N*-resulphonation restores the amide-II absorbance to the higher-frequency/weaker-absorbance situation (Fig. 1d, spectra e, f and g).

In spectra of highly *N*-acetylated heparin (Fig. 1d, spectra h, i and j), the amide-II absorbances occur at a much higher frequency and much lower intensity than expected, suggesting a situation similar to that occurring in native and *N*-resulphonated *N*-desulphonated *O*-desulphated heparins (Fig. 1d, spectra a and g). It is possible that carboxylate-*N*-acetyl interactions, similar to those thought to occur between carboxylate and *N*-

sulphonate groups, occur under these conditions. In accordance with this idea, in heavily *N*-acetylated heparins, the carboxylate symmetrical stretching absorbance occurs at about 1430 cm^{-1} , rather than at a lower frequency. The amount of carboxylate-*N*-acetyl interaction is apparently greater when, after *N*-desulphonation, *N*-acetylation is carried out before, rather than after, *O*-desulphation (Fig. 1*d*, spectra h and i).

It is perhaps relevant that *N*-desulphonated *N*-acetylated heparins resemble native heparin in inhibiting tumour (metastasis-related) heparinase activity (Eldor *et al.*, 1987; Nakajima *et al.*, 1988), and that X-ray analysis of sodium hyaluronate indicates arrays of carboxylate-*N*-acetyl hydrogen-bonding interactions (Guss *et al.*, 1975).

Methyl C—H symmetrical bending vibrations of *N*-acetyl groups at $1382\text{--}1389\text{ cm}^{-1}$ (Figs. 1*d* and 1*e*) show a parallel alteration in intensity, but in the opposite sense, to the amide-II absorbances. Tentatively, we ascribe an absorbance at 1330 cm^{-1} to the amide-II vibration (cf. Walton & Blackwell, 1973); this is much enhanced in artificially *N*-acetylated heparins (Fig. 1*d*, spectra h, i and j and Fig. 1*e*). Changes in this absorbance are broadly in parallel with those of the amide-II bond.

In summary, the results presented accord with a model in which a carboxylate-*N*-sulphonate interaction may occur in native heparin under circumstances dictated by the local environment and chemical fine structure of the polymer. In addition, they indicate that, in artificially *N*-acetylated heparin containing large quantities of *N*-acetyl groups, an interaction between carboxylate and *N*-acetyl groups may occur. An ability of heparins/heparans to move between more than one conformation state, dependent upon local polymer concentrations, pH, ionic strength, the presence and concentration of metal and amine cations and the microstructure of the polymer, may be an important contributory factor to the functions to these polymers.

We thank the Cancer Research Campaign and the Scottish Home and Health Department for financial support and Dr. Ian Sadler and Dr. David Reid of the Science and Engineering

Research Council (S.E.R.C.) service at the University of Edinburgh for carrying out the n.m.r. spectroscopy. C.F.M. was supported by a S.E.R.C. Studentship.

REFERENCES

- Blumenkrantz, N. & Asboe-Hansen, G. (1973) *Anal. Biochem.* **54**, 484–489
- Bruce, J. S., McLean, M. W., Long, W. F. & Williamson, F. B. (1985) *Eur. J. Biochem.* **148**, 359–365
- Cabassi, F., Casu, B. & Perlin, A. S. (1978) *Carbohydr. Res.* **63**, 1–11
- Eldor, M. D., Bar-Ner, M., Yahalom, J., Fuks, Z. & Vlodavsky, I. (1987) *Semin. Thromb. Hemostasis* **13**, 475–488
- Gatti, G., Casu, B., Hamer, G. K. & Perlin, A. S. (1979) *Macromolecules* **12**, 1001–1007
- Grant, D., Long, W. F. & Williamson, F. B. (1987*a*) *Biochem. J.* **244**, 143–149
- Grant, D., Long, W. F. & Williamson, F. B. (1987*b*) *Medical Hypotheses* **23**, 67–71
- Guss, J. M., Huckins, D. W. L., Smith, P. J. C., Winter, W. T., Arnott, S., Moorhouse, R. & Rees, D. A. (1975) *J. Mol. Biol.* **95**, 359–384
- Inoue, Y. & Nagasawa, K. (1973) *J. Org. Chem.* **38**, 1810–1813
- Inoue, Y. & Nagasawa, K. (1976) *Carbohydr. Res.* **46**, 87–95
- Levy, L. & Petracek, F. J. (1962) *Proc. Soc. Exp. Biol. Med.* **109**, 901–905
- Lloyd, A. G., Wusteman, F. S., Tudball, N. & Dodgson, K. S. (1964) *Biochem. J.* **92**, 68–72
- Moffat, C. F. (1987) Ph.D. Thesis, University of Aberdeen
- Nagasawa, K. & Inoue, Y. (1980) *Methods Carbohydr. Chem.* **8**, 287–289
- Nagasawa, K. & Inoue, Y. & Kamata, T. (1977) *Carbohydr. Res.* **58**, 47–55
- Nakajima, M., Irimura, T. & Nicholson, G. L. (1988) *Cell. Biochem.* **36**, 157–167
- She, C. Y., Dinh, N. D. & Tu, A. T. (1974) *Biochim. Biophys. Acta* **372**, 345–357
- Taylor, R. L. & Conrad, H. E. (1972) *Biochemistry* **11**, 1383–1388
- Walton, A. G. & Blackwell, J. (1973) *Biopolymers*, p. 173, Academic Press, New York